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Volume 39 Number 3

ENDOCRINOLOGY

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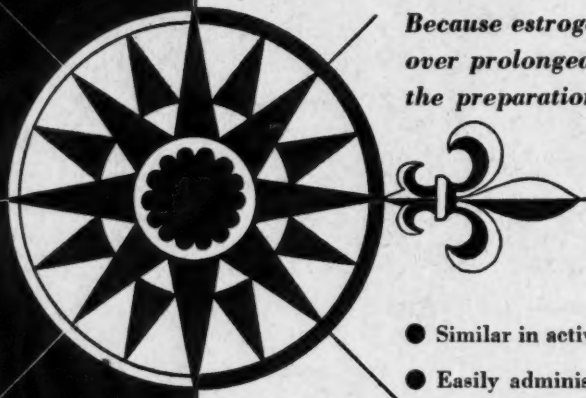

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ENDOCRINOLOGY

VOLUME 39 SEPTEMBER, 1946 NUMBER 3

THE APPLICATION OF THE PRINCIPLES OF STATISTICAL ANALYSIS TO THE BIOLOGICAL ASSAY OF HORMONES[1]

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THE APPLICATION of statistical analysis to the development of methods of assay for the hormones has not been very extensive, especially in the field of anterior pituitary hormones. Most workers have appeared satisfied with a characteristic response or reaction in an animal and the expression of activity in an animal unit, with little regard to the design, dosage-response relationships, or the precision of the method. In this connection, Burn (1930) states "that the field of tame laboratory animals has been nearly exhausted and it remains now for the bolder spirits to discover methods in which a lion or elephant unit may be described."

To some workers the application of statistics to biological assays appears a formidable and even terrifying adventure because they consider the mathematics so involved. Statisticians often have been impractical in the presentation of material and confusing in elaborating symbols and formulae. However, due to the pioneer work of Trevan, Burn, and Gaddum, and more recently Bliss and Irwin, the application of statistics to biological assays has been made in a quite intelligible and practical manner. The publications of these workers show that the application of statistics to biological assays evolves around a few basic principles, substitution in standard formulae, and reference to standard tables. An understanding of the mathematical background is far from essential to the use of statistics, especially as a tool, in devising methods of assay and in studying their precision in order to perform experiments directed towards reducing errors.

Received for publication May 29, 1946.

[1] Presented at the Mont Tremblant Conference on Hormones, September, 1944.

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The application of statistical analysis to biological assays may be compared to the use of a photoelectric colorimeter in studying colorimetric methods of assay. Although a knowledge of photocells and light absorption is very useful in using such a machine, it is far from essential. Also, the use of statistics eliminates subjective impressions as to the interpretation of results in the same manner as the use of a photoelectric colorimeter eliminates subjective impressions in the matching of colors.

In presenting the basic principles necessary to the application of statistics to biological assays, I shall confine myself to the more practical ones, since I am not a statistician and shall have to pass over the theoretical and mathematical considerations.

BASIC PRINCIPLES OF BIOLOGICAL ASSAYS

The first principle is, biological assays must be made on a comparative basis. This means that a standard, often referred to as a reference standard, should be employed and the activity of the sample or unknown compared to this standard under identical experimental conditions. Many workers disregard this fundamental principle or do not appear to appreciate the value of a reference standard. Assays carried out in the absence of a standard are purely qualitative in nature and workers who interpret such assays in a quantitative manner violate a fundamental principle of biological assays, until statistical analysis and experience have shown that the standard is not necessary. Thus far, the only exception to be reported (Bliss and Cattell, 1943) to this principle is in the case of the mortality of eggs of Drosophila melanogaster to x-rays which is used as a biological indicator of dosage.

The essential features of a reference standard are (a) stability, (b) a state or degree of purity in comparison to the substance under test, and (c) the activity capable of being expressed in a given weight or volume. In the development and fractionation of hormone products it is very difficult, at times, to meet these three essential features. The problem of eliminating substances which produce synergistic and antagonistic effects are of considerable importance. Absolute purity is a very desirable feature of a standard, but not essential, and crude preparations identified in terms of total solids, nitrogen content, or other physical or chemical constants, often can serve the purpose until purification has been achieved. In some biological assays the choice of the animal, as well as the standard, is an important consideration. For example, in the assay of estradiol against estrone as a standard, the activity of the estradiol will be different, depending on whether castrated rats or mice are used for the assay. Although these two substances have the same common property and belong to the same group of steroids, different animals utilize them in a different manner. This instance shows the necessity of stating the ani-

mal used in an assay until chemical identification of standard and sample has been achieved.

Many workers consider that, "on the average," small groups of animals respond to a given dose of a hormone in the same quantitative manner each time and feel justified in expressing the activity in animal units without statistical evaluation of the variations. I consider such a procedure is like measuring length by the length of an arm or volume by the handful. Most of us have suffered or gained by the variability of these two methods of measurements at boarding houses so why not apply the results of our experience to biological assays. The establishment of the International Standards has certainly eliminated confusion in assessing the activity of endocrine products. I consider it fair to state that the establishment of these Standards has brought the estimation of the activity of a number of hormone products into the same category as did the establishment of standards of weight and volume to gravimetric and volumetric estimations. It is regrettable that the introduction of International Standards has not followed the clinical use and commercial production of hormone products more closely. The alternative has been the establishment of house standards, working standards or, in a few cases, national standards for comparative testing of products.

The second principle is, the design or plan of the test should permit the determination and use of a dosage response line. This means that a minimum of three dosage levels must be used. A line can be drawn between two points, but it requires at least three points to define the line and to test the fit of the points to such a line. Before proceeding to the quantitative aspects of an assay, qualitative tests must be performed for identification of a hormone. This characteristic reaction must be capable of quantitative measurement and thus serves as the criterion of response. It has been shown for a number of assay procedures that when the doses are administered over a sufficiently wide range that a typical characteristic or sigmoid curve is obtained when the arithmetic dose is plotted against the arithmetic response. Since the response to the doses is not distinguished very well at the ends or flattened portion of the curve, it is obvious that the doses should be arranged so that the responses fall on the steepest portion of the curve. The statistical computations assume that the dosage-response relationship follows a straight line over the dosage range used, and the transformation of the characteristic or sigmoid curve to a straight line is accomplished in different ways, depending, first, on the type of assay and, secondly, on the function of the response which fits the desired relationship.

There are two types of assays (a) those that give all or no responses, often referred to as quantal response assays, and (b) those that produce a graded response, often referred to as the continuous variate type. Typical examples of the quantal response assays are

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the toxicity tests where the animal lives or dies, and the assay of estrogens where the animal shows estrus or does not. This type of assay does not lend itself to degrees of reaction, the response being either positive or negative, and the result is expressed from 0% to 100%. The transformation of the sigmoid curve to a straight line in quantal response assays is achieved by plotting the log of the dose against the probit of the response. Tables have been compiled by Bliss (1935) which give the probit values corresponding to responses ranging from 0% to 100%.

Typical examples of graded response assays are those involving growth of an animal or an organ, such as the assay of the growth hormone or prolactin. In this case the response extends by degrees from a negative value to a maximum value in a numerically continuous manner. The usual method of transforming the dosage-response curve to a straight line in graded response assays is to plot the log of the dose against the arithmetic response. In some cases the log of the response or some other function of the response must be used to fit the desired relationship over the dosage range used. This transformation of a characteristic curve to a straight line is shown very well in the report of Foley et al. (1940) on the assay of prolactin in which the log of the dose is plotted against the (crop weight/body weight)$\times$100. Emmens (1940) has analyzed the data of a number of graded response assays and has shown that in the case of a number of anterior pituitary and gonadotrophic hormones a logistic curve fits the data over a wider range of doses than does the log-dose-arithmetic response relationship. The statistics for the practical use of such curves have not been completed.

The third principle is, biological assays should include a quantitative measurement of the variation. This means that a method of expressing the variation, other than using the average and expressing the range as maximum and minimum values, should be used. The generally accepted method of expressing variation is by means of the standard deviation or some modification of this expression. The calculations involved in determining the standard deviation assume that the variation follows a normal distribution curve.

There are two types of variation encountered in biological assays (a) individual variations, and (b) aperiodic variations. The individual variations are the variations in response of animals of the same species to the same treatment, while aperiodic variation is the time to time variation in response of animals of the same species to the same treatment. Although the same basic principles are involved in computing these variations, the approach to the calculations is different, depending on whether we are dealing with graded response assays or quantal response assays.

The statistics related to quantal response assays have been described by Bliss (1935) and a very excellent practical application of the

use of this method of analysis has been worked out by Miller et al. (1939) for the assay of digitalis. To anyone interested in quantal response assays this is a very good example to follow. The calculations shown can be adapted to any quantal response data and are not limited to the assay of digitalis on frogs.

Since most of the assays dealing with anterior pituitary hormones are of the continuous variate type, I shall show examples of how the data are arranged and explain how the calculations are made for this type of assay; first, by the method of analysis proposed by Irwin (1937), and, secondly, by a method described by Bliss and Marks (1939). I regret that I did not have any data from an assay of anterior pituitary hormones that were suitable to use as an example; instead I have chosen the results from assays carried out on androsterone and testosterone propionate. The method of assay was essentially the same as that described by Gallagher and Koch (1935). Five daily injections of the hormone were given and the increase in length and height of the comb was taken as the response. The same calculations would apply equally well to the data obtained from anterior pituitary hormones or other hormone products producing a graded response.

I considered it would be of some interest to show the arrangement of the data and to explain how the substitution in the formulae was carried out.

CALCULATIONS OF AN ASSAY—IRWIN'S METHOD

The first method of analysis is that described by Irwin (1937). Table 1 shows the arrangement of the data for the calculation of the slope b of the dosage-response line, the ratio M of the potency of the sample to the standard, and the limits of error of the assay. The individual responses and the calculations of the sum of the deviations from the mean squared are shown in Table 2. Turning to Table 1, column 1, it will be observed that 10, 9, and 8 capons were used on the standard and 8, 8, and 8, respectively, on the unknown. The doses of the standard and unknown are listed in column 2, — 60, 120, and 240 gamma for standard and sample, respectively. The logs of the doses are listed in column 3, and in column 4, under nx, the log of the dose x is multiplied by n the number of capons on each dose. The mean of the responses for each dose is listed in column 5, and these values are weighted for numbers by multiplying by the number of capons on each dose. These values are listed under ny. The mean log dose $\bar{x}$ and the mean response $\bar{y}$ are obtained by dividing the sum of nx by the sum of n and the sum of ny by the sum of n, for standard and unknown, respectively.

The next step is to obtain the values for $x-\bar{x}$ for the standard and sample, that is, the difference between the log dose x and the mean log dose $\bar{x}$. These values are listed under column 7 with recognition as to sign; these values are squared and listed under $(x-\bar{x})^2$ in

TABLE 1. CALCULATIONS OF POTENCY AND LIMITS OF ERROR

Method of Irwin, J. Roy. Stat. Soc. 4, No. 1, 1937

No. of capons n	Dose in r	Log dose x	Weighted log dose nx	Resp. $L+H$ y	Weighted response ny	$x-\bar{x}$	$(x-\bar{x})^2$
			Standard				
10	60	1.7782	17.7820	4.2	42.0	−.2898	.08398
8	120	2.0792	16.6336	6.1	48.8	+.0112	.00012
9	240	2.3802	21.4218	10.6	95.4	+.3122	.09747
27	Mean log dose $=\bar{x}_s=$		2.0680	Mean resp. $=\bar{y}_s=$	6.90		
			Unknown				
8	60	1.7782	14.2256	4.5	36.0	−.3010	.09060
8	120	2.0792	16.6336	7.1	56.8	.0000	.00000
8	240	2.3802	19.0416	10.6	84.8	+.3010	09060.
24	Mean log dose $=\bar{x}_u=$		2.0792	Mean resp. $=\bar{y}_u=$	7.40		

$n(x-\bar{x})^2$	$ny(x-\bar{x})$
.83980	−12.17160
.00096	+ .54656
.87723	+29.78388
$S=1.71799$	$S=18.15884$
.72480	−10.83600
.00000	.00000
.72480	+25.52480
$S=1.44960$	$S=14.68880$

$$b_s=\frac{Sny(x-\bar{x})}{Sn(x-\bar{x})^2}\quad\frac{18.15884}{1.71799}=10.56981$$

$$b_u=\frac{Sny(x-\bar{x})}{Sn(x-\bar{x})^2}\quad\frac{14.68880}{1.44960}=10.13300$$

$$b_c=\frac{18.15884+14.68880}{1.71799+1.44960}=10.36991$$

$$M=\bar{x}_s-\bar{x}_u+\frac{\bar{y}_u-\bar{y}_s}{b_c}=2.0680-2.0792+\frac{7.40-6.90}{10.36991}=+.0370$$

$$M=\text{Log}\frac{\text{potency of Unknown}}{\text{potency of Standard}}=+.0370\quad\text{Antilog}=1.089$$

With the Standard at 100% the sample $=108.9\%$

Calculations of Limits of Error

Sum of deviations from mean squared, Standard $=34.72$
Sum of deviations from mean squared, Unknown $=28.76$

$S(d)^2\quad 63.48$

$$\sigma^2=\frac{S(d)^2}{n-r}=\frac{63.48}{51-6}=1.41066$$

$$\sigma^2b_c=\frac{\sigma^2}{Sn(x-\bar{x})_s^2+Sn(x-\bar{x})_u^2}=\frac{1.41066}{3.16759}=.44534$$

$$E^2=\sigma^2\left(\frac{1}{n_s}+\frac{1}{n_u}\right)=1.41066\left(\frac{1}{27}+\frac{1}{24}\right)=.11102$$

$$S_m=\sqrt{\frac{E^2}{b_c^2}+\frac{(\bar{y}_u-\bar{y}_s)^2\sigma^2b_c}{b_c^4}}=\frac{.11102}{(10.36991)^2}+\frac{(.50)^2(.44534)}{(10.36991)^4}=.03213$$

Log S_m at $p=0.95=.03213\times1.96=.06297$
Antilog of $.06297=1.156$, in per cent 115.6
Antilog of recip. $(\bar{1}.93703)=.865$, in per cent 86.5
Limits of Error $=86.5$ to 115.6%
Range of Potency $=(86.5)(1.089)=94.2\%$
$=(115.6)(1.089)=125.9\%$

This sample would be reported as 109% of the standard and 95 chances out of 100 the true potency is not less than 87% or more than 116% of this value or the range of potency is 94% to 126%.

column 8. Squaring $(x-\bar{x})$ makes it a positive value. The values for $(x-\bar{x})^2$ are multiplied by n the number of capons on each dose and the values for $x-\bar{x}$ are multiplied by ny for each dose. The sum of $n(x-\bar{x})^2$ and $ny(x-\bar{x})$ are obtained and substituted in the equation for b shown at the right. The value for b is calculated for the standard and unknown. The bc or b composite is now computed as shown.

The next step is to obtain the value of M, the ratio of the potency

of the sample to the standard. The formula for this is shown with the values substituted. This value is a logarithmic number and referring to the anti-log the value 1.089 is obtained. This means that the unknown is 1.089 times stronger than the standard or in per cent 108.9. If the doses for the unknown were made up from a solution labelled 200 mg. per cc. we would obtain the potency of the original solution by multiplying 1.089 by 200 = 217.8 mg. per cc. Where the sign of M is positive the unknown is more potent than the standard and where it is negative the sample is less potent than the standard.

The next step is to calculate the limits of error of the assay. The

TABLE 2. CALCULATIONS OF SUM OF DEVIATIONS FROM MEAN SQUARED

Data from assay

60 gamma				*120 gamma*				*240 gamma*			
No.	Resp.	d	d^2	No.	Resp.	d	d^2	No.	Resp.	d	d^2
					Standard						
52	3	1.2	1.44	31	6	.1	.01	91	9	1.6	2.56
11	4	.2	.04	89	6	.1	.01	19	11	.4	.16
76	4	.2	.04	23	6	.1	.01	3	12	1.4	1.96
80	4	.2	.04	42	7	.9	.81	83	12	1.4	1.96
35	4	.2	.04	97	7	.9	.81	13	8	2.6	6.76
17	5	.8	.64	15	5	1.1	1.21	47	13	2.4	5.76
14	4	.2	.04	24	5	1.1	1.21	5	11	.4	.16
20	4	.2	.04	37	7	.9	.81	65	8	2.6	6.76
67	5	.8	.64					38	11	.4	.16
22	5	.8	.64								
Total	42		3.60		49		4.88		95		26.24
Mean	4.2				6.1				10.6		
					Unknown						
52	4	.5	.25	50	7	.1	.01	83	12	1.4	1.96
11	4	.5	.25	63	7	.1	.01	19	10	.6	.36
31	5	.5	.25	23	8	.9	.81	30	11	.4	.16
76	4	.5	.25	22	8	.9	.81	13	9	1.6	2.56
67	5	.5	.25	14	5	2.1	4.41	38	10	.6	.36
42	4	.5	.25	89	7	.1	.01	3	9	1.6	2.56
28	5	.5	.25	37	7	.1	.01	87	10	.6	.36
80	5	.5	.25	44	8	.9	.81	97	14	3.4	11.56
Total	36		2.00		57		6.88		85		19.88
Mean	4.5				7.1				10.6		

Sum d^2 standard = 3.60 + 4.88 + 26.24 = 34.72
Sum d^2 unknown = 2.00 + 6.88 + 19.88 = 28.76
Resp. = Response = Increase in length + height of comb.

calculations for the sum of the deviations from the mean of the individual responses are shown in Table 2. These are brought forward and are listed in Table I. The arithmetic is simplified by leaving these values as squares. The σ^2 is obtained by dividing $S(d)^2$ by $n-r$ where n = total number of capons and r the number of doses in this case $51-6 = 45$.

The next step is to calculate $\sigma^2 b$ and E^2 by substituting the values in the formulae shown. The value for E^2 represents the standard deviation squared of the mean response. This is often referred to as the variance of the mean response. We now have the data for calculating the standard error of the assay. The values are substituted in the formula as shown and, depending on the degree of certainty desired, the standard error is multiplied by the usual values employed,—1.96 or 2.576. Multiplying the standard error by 1.96 gives chances of 95

times in 100 and by 2.576 gives chances of 99 times in 100. Most workers are satisfied with 1.96 times the standard error. This degree of certainty is often expressed as p at 0.95. The anti-log of the standard error is referred to for the upper limit 1.156 and the reciprocal of the anti-log for the lower limit 0.865. These values are usually expressed as a percentage, i.e. 86.5 to 115.6. The range of potency is obtained as shown. From the results of this assay, disregarding the decimals as they are undoubtedly not significant, we can say that the best estimate of potency for this unknown is 109 per cent of the standard, and 95 chances out of 100 the true potency is not less than 87 per cent of this value or more than 116 per cent of this value, or the range of potency is 94 to 126 per cent of the standard. A basic principle in the application of statistics to biological assays is that the design or plan of the assay permits complete randomization of the animals and doses. For example if the capons on the standard were near a window and the capons on the sample in a dark corner the environmental conditions would undoubtedly influence the results. An indication of the non-randomization and or non-homogeneity of the animals or doses is the lack of parallelism between the slopes of the standard and sample. The conventional method of testing for parallelism of the regression lines and hence the validity of the assay is by means of the chi square test

$$X^2_b = \frac{(b_s - b_u)^2}{\sigma^2 b_s - \sigma^2 b_u}$$

Where b_s and b_u is the slope of the standard and sample and $\sigma^2 b_s$ and $\sigma^2 b_u$ is the variance of the standard and sample respectively. This equation may be applied to the data shown in table 1 in the following manner:

$$\sigma^2 b_s = \frac{\dfrac{S(d)^2}{n-r}}{Sn(x-\bar{x})^2} = \frac{\dfrac{34.72}{24}}{1.71799} = .84207$$

$$\sigma^2 b_u = \frac{\dfrac{S(d)^2}{n-r}}{Sn(x-\bar{x})^2} = \frac{\dfrac{28.76}{21}}{1.44960} = .94476$$

$$X^2_b = \frac{(10.56981 - 10.13300)^2}{.84206 + .94476} = .10678$$

Referring to X^2 tables (Fisher and Yates, 1938) with n at 1 and p at 0.05 it is seen that 0.10678 is well below the level 3.841 the critical value for significance. The X^2 test is also useful in determining whether

a series of regression lines are of uniform slope and the justification of using a standard value for b in an assay.

The factors which influence the precision of the assay may be seen from the equation for the standard error. They are (1) the slope of the dosage-response line b, (2) the variations in response σ^2, (3) the number of animals n, and (4) the difference in response between the standard and sample. Hence, using the same number of animals per assay, anything that can be done to increase the slope of the dosage-response line or decrease the individual variations of a test will increase its precision. It is only when the mean response of the standard and sample are far apart that the second half of the equation for the standard error contributes a significant effect to the error of the assay.

Irwin's method of calculating the potency and limits of error are relatively simple and straightforward. The method permits considerable flexibility in design and is applicable to a number of assay methods of the graded response type. The doses do not have to be spaced at a constant interval, as I have shown here, but may be spaced in a manner that suits the conditions of the method. The spacing at a constant interval simplifies the preparation of the doses and, to some extent, the arithmetic. The method has the drawback that there is no means of segregating the various factors which contribute to the error of the test.

THE CALCULATIONS OF AN ASSAY—BLISS AND MARKS' METHOD

This method of statistical analysis is based on the principle of variance and factorial analysis. The details of the method have been described by Bliss and Marks (1939) where it is applied to the assay of insulin, and another excellent example of its application has been worked out by Morrell (1942) for the assay of vitamin B_1. The example chosen, Table 3, is an assay of testosterone propionate on the capon by the method of Gallagher and Koch (1935). Five capons were used on each of the three doses,—20, 40, and 80 gamma of testosterone propionate for the standard and sample, respectively. The individual responses are listed under their respective doses in the table at the top of the page. The log of the doses are not entered here but were chosen at an equal logarithmic interval. The log. of the doses are 1.3010, 1.6021, and 1.9031, respectively, and the logarithmic interval is 0.3010. The individual responses are summed for each dose and the sums are squared. These values are entered under the respective doses. In the table at the right the individual responses are squared and summed.

The next step is calculating the correction term according to the formula shown. The correction term is subtracted from the total sum of the squares for doses and the sum of the squares between doses over the number of capons on each dose. The data are now available for the analysis of variance. This is shown in the table under this heading. The degrees of freedom are always less by one than the num-

ber of observations. The assay may be looked upon as a comparison of the responses of 30 capons, some under the same and others under different treatment, that is, the doses of the standard and sample. Since it is a comparison one capon is chosen as a basis and only 29 observations remain to be compared, thus giving 29 degrees of free-

TABLE 3. CALCULATION OF POTENCY AND LIMITS OF ERROR

Method of Bliss and Marks, Quart. J. Pharm. and Pharmacol., #12, 182, 1939

	Standard			Sample				Squared					
Dose	20	40	80	20	40	80							
	6	12	19	6	12	16		36	144	361	36	144	256
	6	11	14	6	11	18		36	121	196	36	121	324
	5	12	14	6	12	19		25	144	196	36	144	361
	6	10	15	7	12	16		36	100	225	49	144	256
	7	7	14	4	10	15		49	49	196	16	100	225
Sum =	30	52	76	29	57	84	=328	182	558	1174	173	653	1422 =4162
Square	900	2704	5776	841	3249	7056	=20526						

$$\text{Correction term} = \frac{(\text{sum of responses})^2}{\text{number of responses}} = \frac{(328)^2}{30} = 3586$$

$$\text{Sum of squares} = 4162 - 3586 = 576$$

$$\text{Between doses} = \frac{20526}{5} = 4105 - 3586 = 519$$

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	
Total	29	576	19.86	
Between doses	5	519	103.80	
Within doses	24	57	2.37	$s = 1.541$

Factorial Analysis

Effect	S_1	S_2	S_3	U_1	U_2	U_3	$NS(x)^2$	$S(xyp)$	$\frac{S^2(xyp)}{NSx^2}$	$\sqrt{\ }$	F
Sample	−1	−1	−1	+1	+1	+1	30	+12	4.80	$2.19 = D$	2.0
Slope	−1	0	+1	−1	0	+1	20	101	510.05	$22.59 = B$	215.2
Parallel	+1	0	−1	−1	0	+1	20	9	4.05		1.7
Curvature	+1	−2	+1	+1	−2	+1	60	1	.02		.0
Opp. Curvat.	−1	+2	−1	+1	−2	+1	60	3	.15		.0

$$M = \frac{KID}{B} \qquad M = \log \frac{\text{potency of unknown}}{\text{potency of standard}}$$

$K = 1.6330$ for 3 dose assay

$I = \log$ dose interval $= .3010$

D & B = from table of factorial analysis

$$M = \frac{1.633 \times .3010 \times 2.19}{22.59} = +.0476 \quad \text{Antilog} = 1.115$$

With the standard at 100% the sample = 111.5%

$$\text{Limits of Error} = Sm = \frac{sKI\sqrt{D^2 + B^2}}{B^2} = \frac{1.541 \times 1.633 \times .301\sqrt{4.80 + 510.05}}{510.05}$$

$$= .03369$$

Log Sm at $p = 0.95 = .03369 \times 1.96 = .06603$

Antilog $.06603 = 1.164$ in per cent $= 116.4$

Antilog of recip. $= 1.93397 = .859$ in per cent $= 85.9$

Limits of Error = 85.9% to 116.4%

Range of Potency $= (85.9)(1.115) = 95.8$

$= (116.4)(1.115) = 129.8$

This sample would be reported as 111.5% of the standard and 95 chances out of 100 the true potency is not less than 86% or more than 116% of this value or the range of potency is 96% to 130%.

dom. Similarly, for doses there are 5 degrees of freedom. Within doses is the remainder. The mean square is obtained by dividing the sum of the squares by the degrees of freedom. The mean square within doses represents the remaining variation when that caused by the different doses is removed. It therefore represents the experimental error and the square root of this value gives the standard error of the individual responses $s = 1.54$. It will be noted that these calculations are merely another way of calculating the standard deviation.

The factorial analysis part of the calculations is a very important one because this separates the different factors contributing to the error of the assay. In the first column the different effects are listed which we wish to separate—sample, slope, parallelism, curvature, etc. The polynomial coefficients shown here are for a three-dose assay and the coefficients to use for a two-dose or four-dose assay are listed in tables (by Fisher and Yates, 1938; Bliss and Marks, 1939). The values for $NS(x^2)$ are obtained by squaring each of the coefficients and multiplying the sum of the squares by the number of animals on each dose. For example, in the first treatment effect, for difference between samples -1^2, -1^2, -1^2, $+1^2$, $+1^2$, $+1^2$ $=6\times5=30$. This is done for each of the effects. The values for $S(xyp)$ are obtained by multiplying the sum of the responses by the polynomial coefficients and adding with due regard to sign. For example, (-1) (30), (-1) (52), (-1) (76), $(+1)$ (29), $(+1)$ (57), $(+1)$ (84) $=+12$. The next step is to square the $S(xyp)$ value and divide it by $NS(x^2)$. These results are listed under the appropriate heading. The square root of the values for sample and slope give the values for D and B. These values represent the square root of the treatment variance for sample and slope. It is essential that both D and B be given the same sign as the sum of the products from which they are computed. The variance ratio F is obtained by dividing the variance attributed to each treatment effect by the mean square for experimental error. For example, $4.80/2.37=2.0$. This is done for each of the treatment effects. Reference to tables in Fisher and Yates (1938) at the 5% points shows whether the value is significant. In this case, with 24 degrees of freedom, a ratio greater than 4.2 is significant. The ratio is determined in this way for each of the effects and from these results one can determine (1) if there is a significant difference between standard and sample, (2) if there is a real dosage-response relationship, (3) if the dosage-response line for the standard is parallel with the dosage-response line for the sample, and (4) if the dosage-response line shows curvature or opposed curvature. These results show there is not a significant difference between sample and standard. There is a positive dosage-response relationship indicating that the range of doses used are on a useful portion of the curve. The lines are parallel and they show an insignificant amount of curvature and opposed curvature. In other words the points fit a straight line over the dosage range used.

The next step is calculating the potency and limits of error. The formula for calculating the ratio of potency of the sample to the standard is $M=KID/B$ where $K=1$, 1.633 and 2.236 for a 2, 3, and 4-dose assay, respectively, see Bliss and Marks (1939). I is the log-dose interval in this case .3010 and D and B are obtained from the table of factorial analysis. When the sign of M is positive the sample is more potent than the standard and when it is negative the sample is less

potent than the standard. Hence, it is important to notice the sign of $S(xyp)$ for sample and slope and in turn the sign of D and B. The value for B in these calculations does not represent the same value for the slope as is obtained in Irwin's method. The values are substituted in the formula as shown, and the resulting ratio calculated.

The calculations for the limits of error follow from the equation shown. The only new term introduced here is s. This is the value obtained in the analysis of variance. The remaining calculations are the same as those described previously for Irwin's method.

This method of analysis lends itself to analyzing many of the variables affecting an assay. For example, the effect of body weight,

TABLE 4. THE EFFECT OF THE NUMBER OF INJECTIONS OF OESTRONE ON THE SLOPE OF THE DOSAGE RESPONSE CURVE AND LIMITS OF ERROR OF THE ASSAY

No. of Injections	Slope b_c	Maximum limits from true potency as per cent 95 times in 100 with responses between 20% and 80% using per assay		
		40 rats	80 rats	160 rats
1	4.50	64 to 157	73 to 138	79 to 126
2	6.66	74 to 135	81 to 124	86 to 116
3	6.60	74 to 135	81 to 124	86 to 116
4	8.48	79 to 127	84 to 118	89 to 113
6	9.35	81 to 124	86 to 116	90 to 111
9	8.15	78 to 128	84 to 119	88 to 113

initial comb size, etc., on the variance may be determined. Time does not permit me to go into these calculations and anyone interested can find examples worked out by Bliss and Marks (193), Morrell (1942), and Bliss and Rose (1940).

The limits of error found in a single assay as above are not necessarily the true limits of the method of assay. They represent the limits under a specific set of conditions and will vary from time to time. They should be regarded as an estimate of the limits of error of the method. The interpretation usually placed on limits of error of an assay is that in a proportion p of assays similar to the one performed the true value will be between the limits found. The range of the true value will vary from one experiment to another because the observed mean value varies, and the factors influencing the range differ in each test.

In order to show the usefulness of statistical analysis in biological assays I have taken two examples from my records. The first, Table 4, is from the assay of estrone by Pugsley and Morrell (1943). This shows the effect of dividing the doses on the slope of the dosage-response curve and on the precision of the assay using different numbers of rats. It is seen that when the total dose is given in one injection the value for $b=4.50$, two divided injections 6.60, and six divided injections 9.50. It is seen that doubling the value of b has a greater

TABLE 5. THE VARIANCE RATIOS USING THE LOGARITHM OF THE DOSE AND THE ARITHMETIC RESPONSE AND THE LOGARITHM OF THE DOSE AND THE LOGARITHM OF THE RESPONSE

	Variance ratio Log. dose—Arith. response	Variance ratio Log. dose—Log. response
Sample	114.83	94.16
Slope	1326.09	1140.28
Parallelism	14.38	.18
Curvature	23.21	.07
Opposed curvature	.23	.00

effect in reducing the limits of error than doubling the number of rats per assay. From these results it is seen that one can plan an assay in accordance with economy of material, time, and effort and precision desired. Figures 1 and 2 and Table 5 are taken from some work on the assay of Vitamin A (Pugsley, et al. 1944) by the vaginal smear method. In Figure 1 the log of the dose is plotted against the arithmetic response and it is seen that the dosage-response relationship is definitely not a straight line. In Figure 2 the log of the dose is plotted against the log of the response and here the desired straight line relationship is obtained. The vertical lines are 1.96 times the standard error of the mean response for each dose. These results

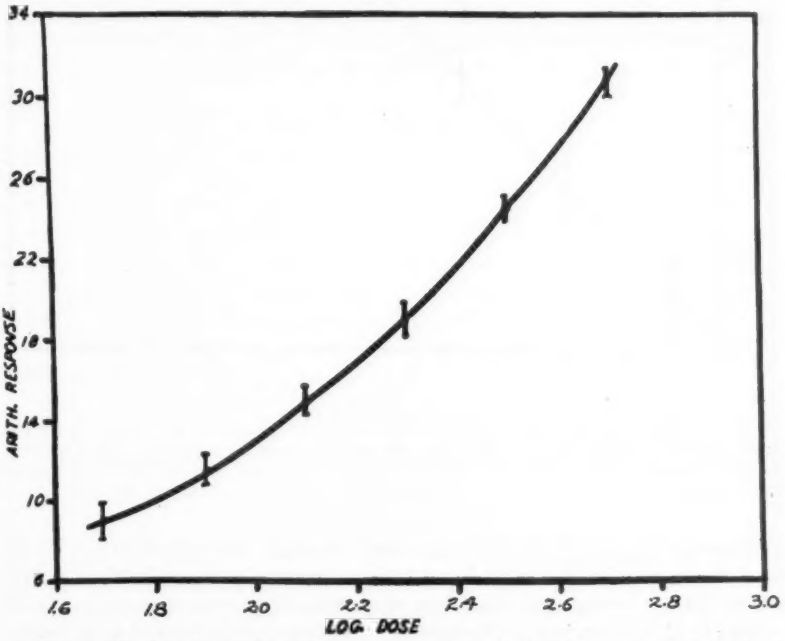

FIG. 1. The log dose-arithmetic response relationship of the assay of Vitamin A by the vaginal smear method. The vertical lines above and below the points are 1.96 times the standard error of the mean response. Note the curvature to the dosage-response relationship.

were obtained with the Canadian Standard cod liver oil and, for the purpose of the calculations, the first, third, and fifth doses were taken as the standard and the second, fourth, and sixth doses as the sample. In the analysis of variance, Table 5, the variance ratios are calculated for the log dose-arithmetic response relationship and the log dose-log response relationship. It is seen that on the basis of log dose-

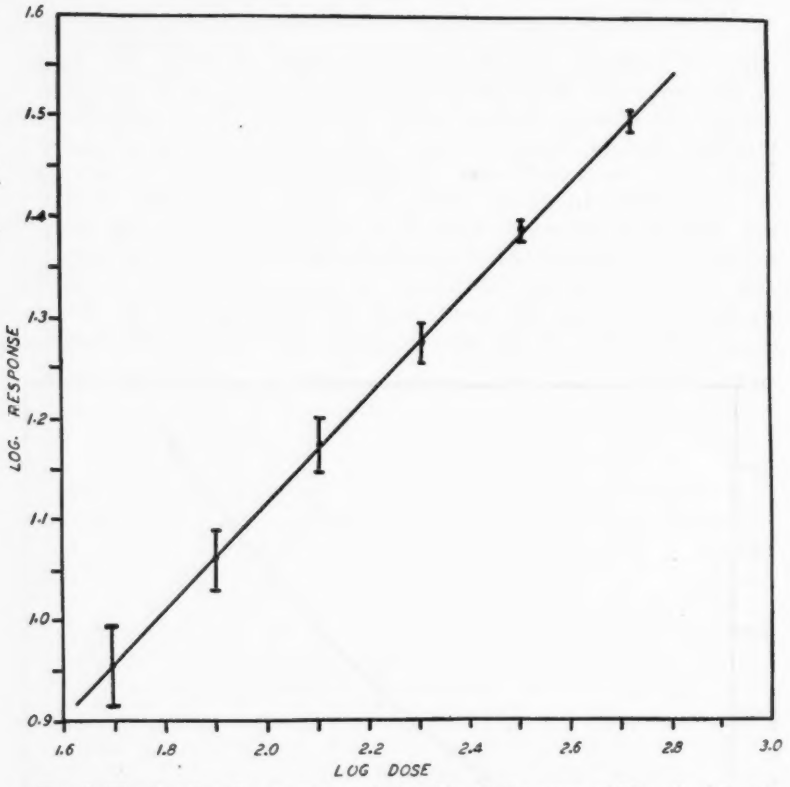

FIG. 2. The log dose-log response relationship of the assay of Vitamin A by the vaginal smear method. The vertical lines above and below the points are 1.96 times the standard error of the mean expressed as logarithms. Note the straight line dosage-relationship.

arithmetic response the lines are not parallel, variance ratio = 14.38 and there is a significant curvature, variance ratio = 23.21. On the other hand, using log dose-log response, the variance ratio, or F value, is at a minimum. The above results were confirmed on a number of assays and the relationship was definitely established as a log dose-log response relationship over a comparatively wide range of doses.

In conclusion, I shall sum up the principles pertaining to a satisfactory method of biological assay:

1. The use of a reference standard with every test.
2. The determination and use of a dosage-response line during every test.
3. The determination of the variations by calculating the standard deviation, or some modification of it, of the response.
4. Careful pharmacological technique. Statistics cannot help careless and faulty technique. At times it can reveal errors in technique.

With these principles in mind and knowing the number of animals, one can calculate the potency and limits of error of an assay regardless of whether the response produced by the standard and sample are equivalent or not. The calculations are a bit tedious but an understanding of the mathematical background is not essential. It is simply a matter of applying formulae and of reference to standard tables.

Statistical analysis of the results of assays obtained in the development and manufacture of hormone products is of considerable importance. It enables one to know which method of extraction gives the best yield per gram of tissue. Often a difference, for example, of 10 per cent in yield is significant in the production of a product. It is only by statistical treatment of assay results that one can ascertain when such a difference is significant. There would be no point in changing a method of extraction if the assay method could not detect the difference.

In the control of the labelled potency of hormone products statistical analysis of the results tell us whether our results differ from the labelled potency by a significant amount. In this way it gives a rational basis for taking official action.

Using statistics as a tool, one is no longer justified in using hit or miss methods or working in the dark not knowing the magnitude of the errors of the test. The errors of biological assays are usually quite large but an estimate of these errors can be obtained which enables one to have confidence in results and a sense of satisfaction that is not otherwise possible.

ACKNOWLEDGEMENT

I am indebted to Dr. C. A. Morrell for his many helpful suggestions and criticisms of this manuscript.

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ESTROGEN IN LATE PREGNANCY MARE SERUM AND OVARIAN INHIBITION

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THE SERUM OF MARES in early stages of pregnancy contains large amounts of a gonadotrophin but in late pregnancy the serum produces ovarian inhibition when injected into immature female rats (Cole and Hart, 1930). Furthermore, during late pregnancy, the ovaries of the mare are atrophic structures (Cole, Howell and Hart, 1931). Studies reported herein indicate that estrogens are responsible for the inhibition.

EXPERIMENTAL

Antigonadotrophic activity of late pregnancy mare serum. The unusually high gonadotrophic activity of early pregnancy mare serum introduces the possibility that the inhibitory effect of late pregnancy serum may be due to the appearance of an antigonadotrophin. This possibility was tested by comparing the ovarian response of equine gonadotrophin alone with equine gonadotrophin plus late prenancy serum. The late pregnancy serum used was tested for its ability to produce ovarian inhibition when given alone. All materials were injected subcutaneously. When equine gonadotrophin and late pregnancy serum were given simultaneously they were injected on opposite sides. The rats were sacrificed 96 hours after the initial injection. A representative example is shown in table 1. The data indicate that, although the serum has an inhibitory effect upon ovarian development when given alone, it does not appreciably depress the ovarian response to injected gonadotrophin. Østergaard (1942) has shown that 0.25 cc. of blood serum from a rabbit immunized against equine gonadotrophin will inhibit an amount of equine gonadotrophin producing 80 mg. ovaries in the rat. Thus, since 30 cc. of late pregnancy serum had no apparent effect, it is evident that very little antigonadotrophic activity is present.[1]

Received for publication July 8, 1946.

[1] Tests for androgen and lactogenic hormone in late pregnancy serum were likewise negative. Fifty cc. of serum injected into immature male rats over a five-day period had no effect upon the seminal vesicles. Forty cc. injected into squabs over a four-day period produced no development of the crop gland.

OVARIAN INHIBITION AND STIMULATION WITH ALPHA ESTRADIOL BENZOATE[2]

Next, we proceeded to find if the ovarian inhibition was produced by the small amount of estrogen in the blood. For purposes of comparison the ovarian response to alpha-estradiol benzoate was first determined (table 2). Ovarian inhibition was produced by $\frac{1}{4}$ and $\frac{1}{2}$ R. U. daily whereas a daily dosage of 1 or more R. U. produced

TABLE 1. TEST FOR ANTIGONADOTROPHIN IN LATE PREGNANCY MARE SERUM

The equine gonadotrophin was injected once daily, the late pregnancy twice daily, over a 3-day period. The rats were 24 to 27 days old at first injection and were sacrificed 96 hours after the initial injection. Littermates were distributed amongst the four groups.

Treatment	No. of rats	Ovarian weight mg.	Uterine weight mg.	Ave. no. of ripe follicles or corpora lutea
Controls—None	9	17.7	46.0	None
30 cc. Late Pregnancy Serum	8	13.7	67.5	None
30 cc. Late Pregnancy Serum plus 12 I.U. Equine Gonadotrophin	8	26.4	114.0	10.3
12 I.U. Equine Gonadotrophin	8	28.2	117.0	13.0

TABLE 2. OVARIAN INHIBITION AND STIMULATION WITH ALPHA ESTRADIOL BENZOATE

The rats were 21 to 23 days old at first treatment and were injected subcutaneously once daily with autopsy 24 hours after the last injection. At autopsy the rats receiving 6 injections were 27 to 28 days old, those receiving 10 injections 31 to 33. Littermates were distributed amongst the groups.

Daily dose of hormone R.U.	No. of daily injections	Total dose R.U.	No. of rats	No. of rats with estrous smears on 5th day of injection	Wt. of ovaries mg.	No. of corpora or ripe follicles per rat
None*	6	None	8	None	16.9	None
0.5	6	3.0	8	4	12.1	None
2.0	6	12.0	8	8	18.8	3.2
20.0	6	120.0	8	8	21.2	6.0
None*	10	None	6	None	20.4	None
0.25	10	2.5	6	None	15.1	None
1.0	10	10.0	6	6	23.3	1.5
4.0	10	40.0	6	6	24.7	5.8

* Rats treated daily with dose of sesame oil comparable to the amount in which the hormone was dissolved for injection.

ovarian stimulation. One-fourth R. U. daily resulted in estrous smears in only 3 of 6 rats. All rats receiving $\frac{1}{2}$ R. U. daily had vaginal smears consisting exclusively of cornified cells on the day of autopsy but on the fifth day of injection only 4 of 8 showed early estrous smears.

[2] Progynon B.

Thus, ovarian inhibition is produced by a dose just below the amount necessary to produce full estrous smears in all animals on the fourth day after the initial injection. This relation between the vaginal smear picture and ovarian inhibition is comparable to that found with late pregnancy mare serum.

The question arises as to what components of the ovary are decreased below that of control ovaries to explain the decrease in weight. Sectioning of control and inhibited ovaries revealed that the decrease is mainly in the number of follicles 400μ or more in diameter. The number of small and intermediate follicles showed no appreciable change.

As stated above, doses of 1 or more R. U. daily produced ovarian stimulation. Occasionally one finds the growth of many follicles but usually the increase in ovarian weight is due to the formation of corpora lutea.

OVARIAN INHIBITION AND STIMULATION WITH LATE PREGNANCY SERUM

Previous studies had shown that late pregnancy serum would produce inhibition if 30 cc. of serum were injected over a 3-day period. If this inhibition depended upon the small amount of estrogen present (10 cc. of serum daily for 3 days would produce an estrous smear in only part of the injected rats) then one should be able to obtain ovarian stimulation by increasing the dose. It is not practical, however, to introduce the large amount of serum necessary to produce ovarian stimulation and consequently the estrogen was extracted.

The extraction of serum was carried out as follows: Six thousand cc. of late pregnancy serum was extracted in batches of 2000 cc. To 2000 cc. of serum was added 300 cc. of concentrated HCl and the mixture placed on a steam bath for 30 to 120 minutes. After cooling, 2 liters of benzene were added and the mixture stirred for 2 hours. The benzene was taken off and the sludge reextracted with 2 liters of benzene. The benzene extracts were combined and washed with 20 cc. of 20% Na_2CO_3. The benzene was distilled off and the residue taken up in 10 cc. of corn oil (10 cc. = 2000 cc. serum).

The extract was injected subcutaneously once daily into immature rats 21 to 23 days old for 7 days with autopsy 24 hours after the last injection on the 28th to 30th days of age. As shown in table 3, both ovarian inhibition and stimulation were obtained with this extract; the nature of the response depending upon the dosage. With untreated serum, inhibition was obtained with 30 cc. whereas with the extract (A176) 42 cc. serum-equivalent produced stimulation. This would indicate that the extract has greater estrogenic potency than the untreated serum. This, no doubt, is due to the freeing of the estrogen from a combined form as the result of hydrolysis. The greater estrogenic potency after hydrolysis is confirmed by the study of the vaginal

smear. All rats receiving a serum-equivalent of 10 cc. of the extract had estrous smears on the fourth day of treatment, whereas 30 cc. of original serum injected over a 3-day period does not lead to complete cornification. Mühlbock (1937) reports that 80% of the estrogen in the blood of the pregnant mare is in the combined form.

Using either untreated serum or an extract of the serum, the inhibitory effect upon the ovary appears to be related to a definite estrogenic potency.

TABLE 3. OVARIAN INHIBITION AND STIMULATION WITH A176, AN ESTROGENIC EXTRACT OF LATE PREGNANCY SERUM

The rats were 21 to 23 days old at first treatment and were injected subcutaneously once daily for 7 days with autopsy 24 hours after the last injection. At autopsy the rats were 28 to 30 days old. Littermates were distributed amongst the groups.

Total dose serum-equivalent per rat* cc.	No. of rats	No. of rats with estrous smears on 5th day of injection	Wt. of uterus mg.	Wt. of ovaries mg.	No. of corpora or ripe follicles per rat
None	18	None	39	23.7	None
5	6	1	74	18.9	None
10	6	6	177	22.5	None
20	6	6	176	20.5	None
40	7	7	194	24.8	0.3
840	4	4	230	30.4	2.2

* A176 was diluted in corn oil so that the amount injected daily varied from 0.2 to 0.6 cc. depending upon the dose.

OVARIAN INHIBITION AND STIMULATION WITH LATE PREGNANCY MARE URINE

As a further check upon the relation between estrogenic potency and ovarian inhibition it is of interest to determine the response to untreated late pregnancy mare urine which has a much higher estrogenic potency than untreated pregnancy serum. As shown in table 4, there is again a definite relationship to estrogenic potency and the ovarian response—in other words, the urine produces inhibition at lower dosage levels than does untreated pregnancy serum.

DISCUSSION

The data submitted provide evidence that the inhibitory effect of late pregnancy mare serum on ovarian development depends upon the estrogen present. Furthermore, it appears that most of the estrogen of the serum is bound in an inactive form with the consequent result that hydrolysis increases the estrogenic activity of the serum. A serum-equivalent of 5 cc. of the extract gives the same response as 30 cc. of untreated serum. According to Mühlbock's studies, more prolonged hydrolysis would free still more estrogen.

One of the most interesting results of this study concerns the

small amount of estrogen necessary to produce ovarian inhibition, and also the very slight increase in dosage necessary to produce stimulation rather than inhibition. These studies give no clue as to whether estrogen acts directly upon the ovary or indirectly through the pituitary.

Several viewpoints have been expressed on the role which estrogens play in controlling ovarian activity. Moore and Price (1932) cleared up many earlier misconceptions regarding hormone antagonism and put forth the view that gonadal hormones had no direct effect upon the gonads, but, rather, depressed the secretion of gonadotrophin by the pituitary. Later it was shown, however, that estrogens in adequate amounts would produce ovarian stimulation in hypophysectomized rats (Pencharz, 1940; Williams, 1940). Westman (1937) likewise obtained evidence that estrogen had a direct stimulating effect upon the ovary. It is probable that ovarian inhibition pro-

TABLE 4. OVARIAN INHIBITION AND STIMULATION WITH LATE PREGNANCY MARE URINE

The rats were 21 to 23 days old at first treatment and were injected subcutaneously once daily for 10 days with autopsy 24 hours after the last injection. At autopsy the rats were 31 to 33 days old. Littermates were distributed amongst the groups.

Total dose per rat cc.	No. of rats	No. of rats with estrous smears on 5th day of injection	Wt. of ovaries mg.	Ave. no. of corpora or ripe follicles per rat
None	7	None	19.1	None
0.5	7	6	14.5	None
2.0	14	14	26.0	3

duced by chronic administration of estrogen is through depressed gonadotrophic secretion of the pituitary as postulated by Moore and Price.

Fevold (1944) suggested that increasing estrogen in the blood facilitates the secretion of LH and decreases FSH secretion. No one has succeeded in showing that FSH and LH, as extracted from the pituitary, ever appear in the blood. The gonadotrophin found in menopausal or castrate urine is quite similar to that found in the urine of normal men and women (D'Amour, 1939; Leathem and Levin, 1941); a fact not in accord with this theory.

From the evidence at hand, it seems fairly certain that estrogen stimulates the ovary directly over short periods and, conversely, depresses the ovary indirectly by inhibiting the secretion of gonadotrophin by the pituitary. The inhibitory effect produced by very small dosages as shown in the present paper is difficult to interpret. The fact that the ovary may be inhibited or stimulated by physiological doses adds weight to the view that estrogen plays an important role in regulating the estrous cycle—a role, which, no doubt, is complemented by progesterone and possibly other hormones.

CONCLUSION

On the basis of the following evidence, it is concluded that the ovarian inhibition produced by late pregnancy mare serum depends upon the estrogen present:

Late pregnancy serum contains no detectable antigonadotrophin.

Alpha estradiol benzoate produces a comparable ovarian inhibition at the same physiological level (the physiological level being determined by the vaginal smear response).

Like alpha estradiol benzoate, late pregnancy mare serum produces ovarian stimulation rather than inhibition when the dosage is increased.

Ovarian inhibition or stimulation is likewise produced by comparable physiological doses of late pregnancy mare urine.

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THE NATURE OF CIRCULATING ESTROGEN: LIPOPROTEIN-BOUND ESTROGEN IN HUMAN PLASMA[1,2]

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IN THE COURSE of investigations on the activity of the naturally occurring blood estrogens, it was found that the viscera were essential for estrogenic stimulation (Szego and Roberts, 1945). No evidence for estrogenic activity could be obtained in the eviscerated rat under a wide variety of conditions. Apparently, the presence of exogenous estrogen in the circulating blood was not in itself an adequate stimulus. From these and other observations it seemed possible that the estrogen molecule might undergo, under normal conditions *in vivo*, certain changes which endow it with the physico-chemical properties necessary for its activity.

Accordingly, studies on the nature of circulating estrogen in several species were undertaken (Szego and Roberts, 1946). It was shown that approximately two-thirds of the estrogenic activity of whole blood, cells, plasma, and serum occurred closely associated with the blood protein. The fact that this estrogen, as well as the one-third not bound to protein, could readily be made to dialyze quantitatively past a collodion membrane, suggested that it was available for physiological action by dissociation at the cell membrane. The existence of an equilibrium between protein-bound and unbound estrogen in blood was also indicated.

These results on the quantitative distribution of protein-bound and unbound estrogen have been confirmed in more recent unpublished studies of normal citrated plasma, pooled from human subjects. Again, dialysis yielded values for total blood estrogen similar to those obtained by exhaustive chemical extraction. After repeated dialysis of blood obtained from other sources, no remaining estrogen could be

Received for publication July 11, 1946.
[1] This work was supported by grants from the Penrose Fund of the American Philosophical Society, from the Applied Research Foundation, and from G. D. Searle & Co.
[2] The plasma protein fractions were generously supplied by Prof. Edwin J. Cohn of the Department of Physical Chemistry, Harvard Medical School, Boston, Mass They were prepared in cooperation with the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health, from blood collected by the American Red Cross for the Committee on Medical Research of the Office of Scientific Research and Development.

detected in the blood samples. It is of interest to note that significant quantities of estrogen could not be obtained by ultrafiltration of pooled human plasma.

Since an estrogen-protein complex was a reasonable conclusion from the work described above, an investigation was undertaken of the estrogenic activity of purified protein fractions prepared from human blood. Identification of this estrogen was also attempted.

EXPERIMENTAL

1. *Direct assay of plasma protein fractions.*

Through the generous cooperation of Dr. Edwin J. Cohn and his associates, samples of purified human plasma protein fractions were

TABLE I. ESTROGENIC ACTIVITY OF PURIFIED PLASMA PROTEIN FRACTIONS

Sample number	Fraction[1] assayed	Treatment	Equivalent cc. plasma injected per animal[2]	Estriol equivalents (micrograms per 100 cc. plasma)
1	I (fibrinogen)	direct assay[3]	19	0
2	II (γ-globulin)	direct assay[3]	75	0
3	III (β-globulin)	direct assay[3]	42	0
4	III-0 (β-globulin plus lipids)	direct assay[3]	23 to 46	1.7
5	III-0 (β-globulin plus lipids)	dialysis of[4] 0.65% soln.	—	1.5
6	III-0 (β-globulin plus lipids)	dialysis of[4] 8.4% soln.	—	0
7	IV-1 (α-globulin plus lipids)	direct assay[3]	50 to 60	trace
8	IV-3,4 (α- and β-globulin)	direct assay[3]	67	0
9	V (albumin)	direct assay[3]	28	0.1
10	dried plasma (citrated)	direct assay[3]	16	1.8
11	dried plasma (citrated)	dialysis of[4] 6.5% soln.	—	1.8

[1] The numerical designations in this column for the plasma protein fractions are those assigned by Cohn et al. (1946).
[2] Based on the normal content of human plasma for the individual proteins assayed.
[3] In physiological saline. Assayed against standards in saline.
[4] See text for extraction procedure. Standards in olive oil.

made available. These were tested for estrogenic activity by a modified Astwood technique (Szego and Roberts, 1946). Relatively concentrated solutions in physiological saline were injected intraperitoneally in 0.5 to 2.0 ml. amounts. Standards were similarly assayed. In Table I the results are expressed in terms of estriol, since it was subsequently found that the latter was probably the main or sole estrogen present.

Direct assays of dried whole citrated plasma pooled from human subjects gave values approximating 1.5 to 2.0 micrograms of estriol (average 1.8) per 100 ml. of original plasma. Similar assays of the purified lipoprotein fraction, III-O, gave an average value of 1.7 micrograms of estriol, referred to 100 ml. of original plasma. No sig-

nificant estrogenic activity was obtained in the other purified plasma protein fractions, including fraction IV-1 which contains a fair proportion of bound lipid material. Thus, the fractionation procedures of Cohn et al. (1946) selectively concentrate the plasma estrogen into one of the two lipoprotein fractions (III-0). It is to be noted that the estrogenic activity of this material diminishes with time even when stored in the cold. This suggests that some part of the estrogen-protein complex undergoes changes which result in a loss of activity.

In addition, it was found that cold ether extraction of fraction, III-0 in saline removed none of the estrogenic activity. A large proportion of the other lipoid material is apparently extracted by this procedure.

2. *Dialysis of plasma and lipoprotein solutions.*

Dialysis of the original plasma and of fraction III-0 for a period of 48 to 72 hours yielded estrogen values comparable to those obtained by direct assay (Table I, samples 5 and 11). The dried plasma was made up to 6.5 per cent in distilled water, and 100 ml. samples of this solution were dialyzed against distilled water. Portions of the lipoprotein fraction III-0 equivalent to its normal plasma concentration (0.65 per cent) were dissolved in 0.9 per cent sodium chloride and dialyzed similarly. These procedures were carried out in the cold. The dialysates after acid-hydrolysis were extracted with ether and made up in olive oil for assay.

Assuming dialysis to equilibrium, the plasma samples were found to contain about 1.8 micrograms of estriol per 100 ml. of normal plasma. The lipoprotein fraction contained an average of 1.5 micrograms, expressed in the same fashion. Similar results were obtained when these fractions were dialyzed against 0.02 M phosphate buffer at pH 7.1. As before, the dried plasma was dissolved in distilled water. The lipoprotein, however, was made up in the buffer. It is apparent from these results that protein-bound estrogen is readily dialyzable under simulated physiological conditions. Preliminary experiments indicate that dissociation by dialysis of the estrogen-protein complex is inhibited by high salt, and, (see Table I, sample 6), by high lipoprotein concentrations. It is of interest to note that other lipoid substances will dialyze out of fraction III-0 under the conditions noted above. Thus, the dialysates after acid hydrolysis, as well as the concentrated ether extracts of the latter, were of a distinct yellow hue.

3. *Identification of the estrogen in lipoprotein dialysates.*

Several 72 hour dialysates of fraction III-0 (about 1 per cent in distilled water) were pooled, hydrolyzed with acid, and ether-extracted. The extract was dried, taken up in benzene, and extracted with 0.3 M. sodium carbonate as described by Mather (1942).

The carbonate phase (containing any estriol present in the original extract) was acidified, extracted with ether, and made up in olive oil for assay.

The benzene phase was dried and subjected to a modified micro-Girard procedure to separate estrone and estradiol (Szego and Samuels, 1943). The final ether fractions obtained were also made up in olive oil.

Assay of the three samples revealed significant estrogenic activity only in the "estriol" fraction.

DISCUSSION

The data presented above strongly suggest the existence of a specific protein-estrogen complex in human blood. The protein component of this *estroprotein* is probably of the β-globulin type, since it fractionates into fraction III-0 of Cohn. Evidence is presented that the estrogen component of this complex is probably mainly estriol.

The nature of the linkage to protein is such that organic solvents do not readily remove the bulk of the estrogen as it occurs in blood without previous partial hydrolysis of the protein itself (Szego and Roberts, 1946). Similarly, cold ether extraction of purified lipoprotein in saline, while it removes other less firmly bound lipid material, leaves the estroprotein complex intact. Nevertheless, dialysis readily results in the progressive dissociation of this complex, yielding estrogen in an esterified form, from which it can be released by brief acid hydrolysis. These facts indicate that the estrogen is attached to protein in a hydrophilic esterified form. They also may explain the finding that substantially all of the estrogen in plasma is concentrated in the purified lipoprotein fraction, whereas only about two-thirds of plasma estrogen is found in aqueous acetone or alcohol-ether precipitates of plasma protein. Thus, partial dissociation of the complex may occur in aqueous media such as blood. The equilibrium of this dissociation is apparently regulated by salt and protein concentration. Removal of water, however, as during the fractionation of the plasma proteins, may favor reassociation.

No evidence is available concerning the chemical nature of the ester portion of the estroprotein complex. Since sodium estriol glucuronide is found in human urine, the ester involved may be the glucuronide. Unfortunately, the quantities of estrogen in human blood are so small as to make isolation and direct identification of either the estrogen itself, or if its ester component, extremely difficult.

The activity of the estrogen-protein complex appears to be at least equal to that of the free estrogen (Table I). The rôle of the viscera in the apparent activation of injected free estrogen still remains to be clarified. The function of the liver in the elaboration of some plasma proteins, however, suggests that this organ may be the site of formation or combination of the estroprotein complex. This com-

plex, and others similar to it, may be of considerable importance as a steroid transport mechanism.

SUMMARY

Direct assay of the purified protein fractions of human plasma, as prepared by Cohn et al. (1946), revealed that practically all of the protein-bound estrogen present occurs in the lipoprotein fraction III-0. The estrogen was found to dialyze readily out of this fraction under simulated physiological conditions.

Evidence was presented that the estrogen in fraction III-0 may be estriol, and that it is attached to the protein in an esterified form.

The physico-chemical characteristics and physiological significance of the estrogen-protein complex have been discussed.

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FLUCTUATIONS IN WEIGHT, HEMATOCRIT AND PLASMA PROTEIN WITH THE MENSTRUAL CYCLE

D. N. DANFORTH, P. K. BOYER, AND SAMUEL GRAFF[1]

IT IS GENERALLY accepted that a significant increase in weight and occasionally manifest edema may occur in some women at about the time of menstruation (Ehrenfest, 1937; Reynolds, 1939; Thomas, 1933; Sweeney, 1934; Atkinson and Ivy, 1936). These have been attributed to a premenstrual retention of sodium which is due to the action of the ovarian hormones (Thorn, Nelson and Thorn, 1938; Greenhill and Freed, 1940; Freed, 1945). A tendancy to increase in blood sodium and chlorides during the midcycle has been reported (Eckstein, 1941); but there is no evidence of premenstrual elevation of these constituents in the circulating blood. More recently, Greisheimer et al. (1946) were unable to demonstrate typical or significant changes in pH, carbon dioxide content, chloride or total base in relation to the menstrual cycle.

With regard to hemoglobin and the formed elements, there appears to be general agreement that no gross changes occur (Smith, 1936; Kracke, 1941). However, it is of interest that a physiologic anemia does occur at the time of menstruation in the pig-tailed macaque (Guthkelche, 1937), and that earlier studies have suggested a similar occurrence in the human being (Smith, 1936).

The present study was undertaken primarily to determine whether any gross blood electrolyte fluctuations could be detected and correlated directly with alterations in weight. It was considered that estimations of hematocrit and protein might serve to clarify any significant changes which occurred.

MATERIALS

Six normal resident nurses were selected. None had significant menstrual prodromes or distress. Weight readings and blood specimens were taken at the same time of day, approximately every three days for one month. Weights were measured with the same scale, with the subjects in the same clothing, after evacuation of the bladder and lower bowel. Fasting blood specimens were drawn without stasis from the basilar vein and heparinized immediately. They were analyzed for hamatocrit (centrifuged one hour),

Received for publication July 19, 1946.

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plasma protein (by the micro Kjeldahl method), sodium (gravimetrically), and total base. The dates of the previous menstrual flow and of the flow during the test period were recorded. Samples were then numbered according to the pre- or post-menstrual day upon which they had been withdrawn. A routine hospital diet was used throughout, without attempt at limitation or assay.

DATA

I

Subject	*Date of last menstrual period*	*Onset of present menstrual period*
A	2/21	3/13
B	2/20	3/21
C	2/23	3/24
D	2/14	3/15
E	2/9	3/11
F	2/10	3/6

II

TABLE I

Subject / Date	A			B			C		
	Weight	Hemat.	Prot.	Weight	Hemat.	Prot.	Weight	Hemat.	Prot.
2/28	122¼	40.4	7.28	128½	36.8	7.64	127	36.8	7.46
3/3	121½	39.1	7.88	127½	35.5	7.81	126¼	37.8	7.69
3/6	123	40.6	7.33	127¾	—	7.08	126¾	38.3	7.75
3/9	123	42.2	7.69	129	—	—	127	39.5	7.94
3/13	122¼	42.5	7.25	130	37.8	7.00	128	—	7.18
3/15	123½	41.4	7.38	128	38.7	7.84	128½	39.7	7.98
3/17	121¾	43.3	7.46	128	38.3	7.66	126¾	40.2	7.41
3/21	121¾	44.0	7.62	128	37.0	7.28	127½	41.5	7.52
3/24	122	41.8	7.33	129½	37.1	7.37	129¾	39.1	7.21
3/27	122	41.6	7.18	129	36.1	7.67	128½	40.1	7.76

Subject / Date	D			E			F		
	Weight	Hemat.	Prot.	Weight	Hemat.	Prot.	Weight	Hemat.	Prot.
2/28	116¾	38.3	7.04	139	43.1	8.00	123½	43.2	7.75
3/3	114½	35.2	7.44	139¾	41.7	7.36	123½	43.1	7.50
3/6	116¼	39.8	7.44	139¾	42.6	7.65	122	42.0	7.76
3/9	115¼	41.9	7.36	140½	44.3	8.31	122¾	41.3	7.50
3/13	118	38.3	7.00	141	42.2	7.83	121¾	42.7	7.70
3/15	116½	39.7	6.83	140¼	41.4	7.84	121½	43.5	7.98
3/17	117½	37.4	6.98	139¾	43.6	8.03	120½	41.4	8.20
3/21	115¼	40.4	7.18	—	—	—	121	43.8	7.39
3/24	115¼	39.9	7.42	139¾	44.3	7.77	121¾	41.9	7.48
3/27	114½	39.6	7.17	140½	45.2	7.68	121¾	—	7.19

Weight is expressed in lbs., hematocrit (cell volume) in per cent, and protein in grams per 100 cc.

III

The data for sodium, potassium and total base are omitted from this communication.

METHOD OF ANALYSIS AND RESULTS

When plotted in the absolute values which were obtained, none of the results showed any significance whatever, either individually or collectively.

An attempt was then made to demonstrate by statistical methods any patterns which might be present but concealed by individual trends. It was also desired to reduce the variables to common terms so that they might be compared with one another. The individual groups of data were analyzed for trend by fitting a straight line to the chronological data by the method of least squares. In cases where

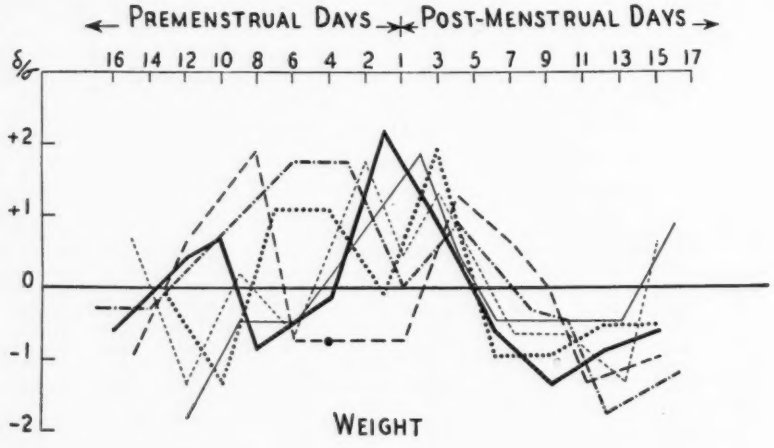

FIG. 1

no trend was observed, fluctuations were computed as deviations from the mean. In instances where significant trend occurred, variations were computed as deviations from the trend line. All deviations were finally expressed in units of the standard deviation of their own series of data, and so recorded upon graphs (Figs. 1–3). Composite curves were fitted by inspection (Fig. 4).

In the instances of sodium, potassium and total base the absolute values were within normal limits. No consistent patterns could be demonstrated, either by gross examination or by statistical methods of analysis. These data are therefore omitted from this communication. Rather constant patterns were found in the cases of the weight and hematocrit, and consistency in the proteins during the postmenstrual period.

It is of interest that all of the subjects showed a significant *upward* trend in *hematocrit* during the period of observation. None showed a

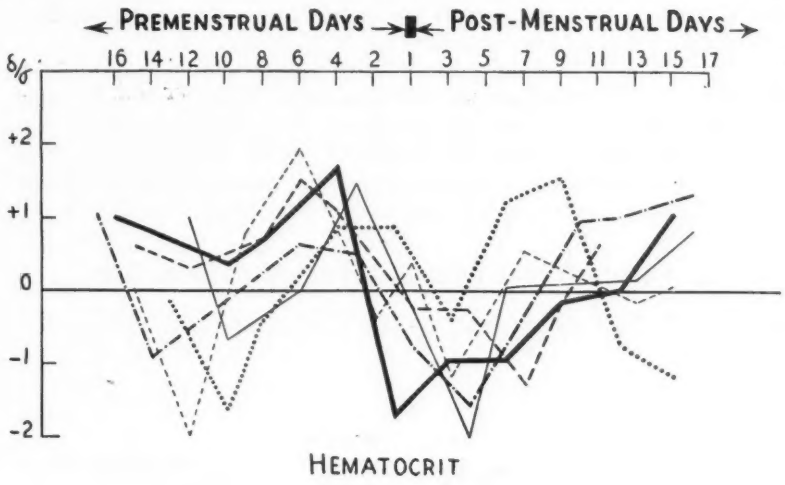

FIG. 2

significant upward or downward trend in weight. In five of six subjects protein showed a downward trend; no protein trend was demonstrable in the sixth. Intermediary swings are shown in the graphs.

COMMENT AND CONCLUSIONS

By a method of statistical interpretation of data, certain patterns in weight, hematocrit and plasma protein have been demonstrated

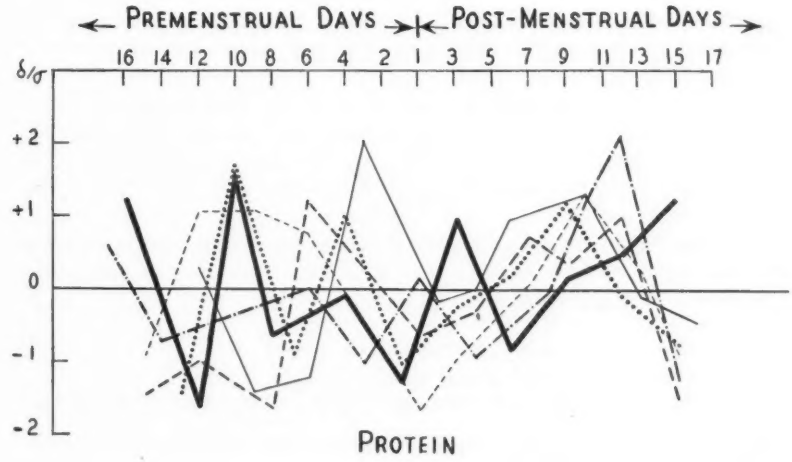

FIG. 3

and correlated with the menstrual cycle. No consistent fluctuations in sodium, potassium or total base could be shown.

In the case of the weight, hematocrit and protein, it is emphasized that the actual determinations were all within the normal range, and showed grossly no statistically significant differences or evident correlations. However, when the individual series were corrected for trend and plotted in the manner indicated, certain portions of the resulting curves showed a correlation which is unusual for short-term

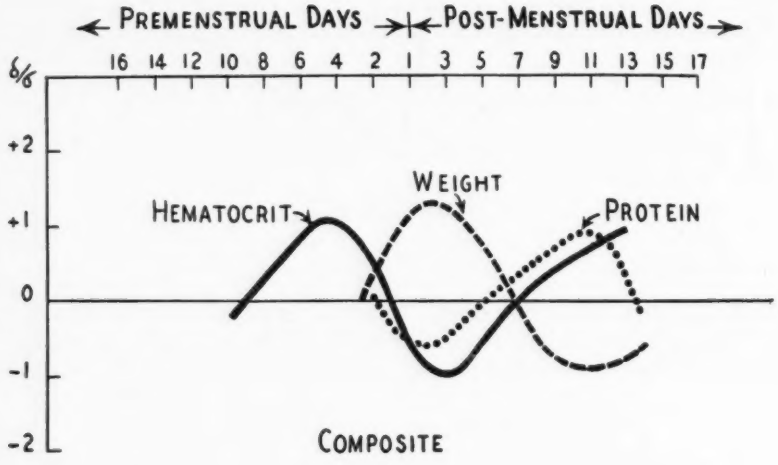

FIG. 4

biological data. For this reason, and since the findings are in agreement with related experimental and clinical data, one may attach to them biological significance.

The elevation of the weight curve at about the time of menstruation is reflected by a simlutaneous decline in hematocrit. As one might expect, the protein curve is similar to that of the hematocrit. The latter curves might suggest a relative increase in blood volume at about the time of menstruation.

Although the required calculations are extensive, the statistical method employed here may be useful as an aid to the interpretation of otherwise obscure chronological data.

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ERRATUM

In the article by Juhn, Mary "Effect of Thiouracil on the Juvenile Plumages of Brown Leghorn Fowl," Endocrinology 39: 14–22. 1946, the order of the legends for the colored figures on page 19 were inadvertently reversed. The legends should read:

Colored films reading from left to right.
Film A) Fig. 1. Normal male breast feather.
Fig. 2. Normal female dorsal feather.
Fig. 3. Thiouracil-modified female breast feather.
Fig. 4. Normal female breast feather.
Film B) Fig. 5. Thiouracil-modified male dorsal feather.
Fig. 6. Normal male dorsal feather.
Fig. 7. Thiouracil-modified male breast feather.
Fig. 8. Thiouracil-modified female dorsal feather.

THE EFFECT OF STEROIDS RELATED TO THE CORTICAL HORMONES AND OF STILBESTROL ON THE ADRENALECTOMIZED GUINEA PIG

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THE ABDOMINAL fibroids which are produced in the guinea pig by estrogens (Lipschütz and Iglesias, 1938) may easily be prevented by the administration of certain cortical steroids such as desoxycorticosterone and dehydrocorticosterone (work of Lipschütz, Nuñez, Vargas and Zañartu; see review by Lipschütz, 1944). Since 28 different steroids have been extracted from the adrenal cortex (Reichstein and Shoppee, 1944) of which 6 or 7 have cortical activity, the question arose as to the role of these steroids in the system of anti-tumoral auto-defense by the maintenance of the steroid balance, in accordance with the concept of Lipschütz (1943).

We proposed to attack this problem by removing the adrenal glands from guinea pigs which were to be treated afterward with fibromatogenic quantities of estrogen. But in order to study the question experimentally, it was necessary to replace the adrenal cortex with a steroid possessing cortical activity but lacking any antifibromatogenic action. Selye (1941) found that acetoxypregnenolone (AOP; Δ^5 pregnene-3,21-diol-20-one-21 acetate) possesses cortical activity, although less than does desoxycorticosterone acetate (DCA). Segaloff and Nelson (1942) confirmed this finding. Cleghorn (1943) found that the cortical activity of AOP in the adrenalectomized dog is 6 times less than that of DCA. On the other hand, AOP was demonstrated to have no antifibromatogenic action in the guinea pig in quantities up to 320 micrograms daily (Lipschütz, Bruzzone and Fuenzalida, 1943) and even 1240 micrograms daily (unpublished results).

With this background we began removing the adrenal glands from

Received for publication July 23, 1946.

[1] This work has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, and The Ella Sachs Plotz Foundation for the Advancement of Scientific Investigation, grants administered by Prof. A. Lipschütz. Steroids have been generously supplied by Dr. E. Oppenheimer, Ciba Pharmaceutical Products Inc., and stilbestrol by Messrs. E. R. Squibb & Sons.

Thanks are due to Prof. A. Lipschütz for helpful criticism and for the revision of the manuscript.

the guinea pigs, treating them with AOP and subsequently adding stilbestrol as a fibromatogenic agent. But first we had to determine the minimum amount of DCA required by the animal in order to compare it later with the requirement of AOP.

METHOD

In the literature at our disposal we have found two techniques for the ablation of the adrenals in the guinea pig (Simmons and Whitehead, 1936; Schachter and Bebbe, 1939). We have performed the operation by a different method in animals weighing more than 400 grams.

The operation was carried out in two stages separated by an interval of at least 8 days. In the majority of cases we began with the *right* gland, since it is the more difficult to remove. The animal, which is fasting in order to reduce the size of the abdomen, is placed in lateral semidecubitus on the side opposite that for operation. The incision in the abdominal wall is made close to and parallel to the costal margin; it is begun 1 cm. below the anterior end of the last rib and is extended 4 or 5 cm. toward the epigastrium. In operating on the right side the assistant separates the intestinal loops and the liver with a compress. The operator retracts the kidney and severs the connections between this organ and the gland by means of a small hook. The gland is then lifted, first with the aid of a hook and later with a pledget of cotton held in forceps, care being taken not to cut the gland. In this way it is separated from the wall and from the liver, remaining attached to the vena cava and to the arterial pedicle. The gland is gently retracted toward the kidney, which permits one to see, near the upper pole, the adrenal vein which empties into the cava. At this point a Pean forceps is introduced, directed caudad touching the gland and enclosing both the great vessel and the adrenal vein. Beginning at the upper pole, the gland is lifted by means of a pledget of moist cotton, which is slid over the edge of the forceps until the adrenal vein is severed. The separation is continued in the same way until the arterial pedicle is reached, which is cut with scissors. A moment later the forceps are withdrawn. If the opening in the cava bleeds it can be held in the forceps or a bit of abdominal muscle may be pressed upon it to stop the hemorrhage.

On the *left* side the gland is of larger size. As in the case of the right gland we use the forceps placed over the renal vein after separating the gland from its connections with the wall and the kidney. We slide the pledget of cotton over the edge of the forceps to sever the gland from the veins and the remaining attachments. We also castrate the females.

The muscles and the skin are sutured with thread.

When the second operation was completed the implantation of steroid pellets was carried out according to the technique of Deanesly and Parkes (1938); the tablets were dried *in vacuo* and weighed before and after their stay in the animal. Pellets of various weights were used to cause different amounts to be absorbed and in this way to determine the minimum requirement of hormone.

The food was that ordinarily used in the laboratory: green alfalfa, carrots and maize *ad libitum*.

In the results to be reported animals which died within 2 days are not included, their deaths being attributed to the operation.

RESULTS

Desoxycorticosterone acetate. In Table 1 are found 32 animals (among them 25 males) divided into 3 groups which received DCA. In none of these was accessory adrenal tissue found at autopsy.

The 11 animals of group I absorbed at most 156 micrograms daily. All died within 12 days. We consider this the maximum period of survival when the animal receives less than the minimum requirement of DCA. Schachter and Beebe (1939) and also Simmons and Whitehead (1936) obtained survival up to 7 days in their untreated adrenalectomized animals; for this reason we attribute the longer survival

TABLE 1. ADRENALECTOMIZED GUINEA PIGS TREATED WITH SUBCUTANEOUS PELLETS OF DCA

Group	Number of animals	Absorption of DCA microgm. per day	Number of animals dying on days indicated						Number of animals dying—days after removal of pellet		Loss of weight per day, gm.	
			4–7	9–12	13–18	21–34	73–95	115–128	5–8	16	Mean	Range
I	11	50–156	6	5	0	0	0	0	—	—	60	20–110
II	14	179–525	3	3	2	2†	2*	2*	—	—	56	0–310**
III	7††	175–336	0	1	4	2	—	—	6	1	172	110–240

* Of these 4 animals, 2 died when DCA became exhausted.
** Omitting the 2 animals mentioned in *.
† 28 and 34 days.
†† Pellets removed after 12, 14, 14, 15, 18, 21 and 24 days.

in our group to the administration of a certain, although insufficient, amount of steroid.

The 13 animals of group II absorbed 179 to 525 micrograms per day. Nevertheless 6 animals died within 12 days in spite of the fact that the absorption of DCA was much greater than 175 micrograms per day and in 1 reached 525 micrograms. Eight animals survived longer than 12 days. Of these 1 died of heat stroke on the 15th day (293 micrograms per day) and another of an initial intercurrent infection on the 73rd day (228 micrograms). In 4 others, death occurred without any recognized cause despite the fact that the animals were in good condition. In 1 which survived for 115 days the absorption was 188 micrograms per day. The others survived 13 to 34 days with absorptions greater than 273 micrograms. In 2 animals which survived 95 and 128 days respectively, death occurred when the pellet was exhausted; the absorption was 241 and 179 micrograms per day. The animal which lived 128 days doubled his body weight in the course of the experiment.

In group III are 7 animals from which the pellet of DCA was removed after 12 days of survival in order to determine whether the survival was due to the cortical steroid or to the presence of accessory adrenal tissue. At the time that the pellet was removed the animals appeared normal and were gaining weight. Nevertheless they died not more than 8 days after the removal of the pellet, with the exception

of 1 which died on the 16th day, perhaps because a fragment of DCA remained and was gradually used up.

Thus it is demonstrated that the survival of groups II and III is due to the DCA absorbed, since no glandular fragments were found.

In the animals which died with a subcutaneous pellet present, and also in those which died after removal of the pellet, general emaciation was found at autopsy, with hyperplasia of the lymphatic system, bile-stained gastric contents with erosions and ulcerations of the gastric wall which perforated the wall in one of the animals, and scanty material in the intestinal tract. Ulcers of the gastric mucosa are a frequent finding in animals which die of adrenal insufficiency (Mann,

TABLE 2. ADRENALECTOMIZED GUINEA PIGS TREATED WITH SUBCUTANEOUS PELLETS OF AOP

Group	Number of animals	Absorption of AOP microgm. per day	Number of animals dying on days indicated					
			2–7	10	14–18	25–35	40–47	153–162
I	8	200–840	6	0	1	0	1*	0
II	4	852–967	1	0	0	1†	1*	1‡
III	9	1000–1250	0	1	2	1*	4*	1‡

* Died when stilbestrol was added.
† AOP removed; died 4 days later.
‡ Killed for necropsy.

1916). We have observed them in all the animals whose death was due to obvious cortical insufficiency, such as those of group III. Emaciation is the most constant sign in the death of animals as a result of adrenal insufficiency. The greatest loss of weight occurred in the animals of group III, i.e. those from which the steroid was removed, and in the 2 animals of group II which died after exhaustion of the DCA.

From the data summarized in Table 1 it is concluded that the majority of our adrenalectomized guinea pigs survived for more than 12 days with 175 micrograms or more per day of DCA. While all of the animals in group I which received 50 to 156 micrograms of DCA per day died in the course of 12 days, only 7 of the 21 animals of groups II and III which absorbed more than 175 micrograms died within 12 days; the remaining 14 survived. The greater absorption, however, is no guarantee of a prolonged survival. The 4 animals of group II which survived more than 70 days absorbed 179 to 241 micrograms per day, while those which absorbed a greater amount had a survival of less than 34 days.

Acetoxypregnenolone. The results are summarized in Table 2. In none of the 21 animals (of which 15 were males) was accessory adrenal tissue found.

In group I, out of 8 animals which absorbed between 200 and 840

micrograms, 6 died within 7 days as a result of cortical insufficiency. This period of survival is less than when the animal receives an insufficient amount of DCA (see our results in Table 1) and is in agreement with the observations of the authors mentioned above in untreated adrenalectomized guinea pigs. With amounts of AOP greater than 700 micrograms per day, however, some animals managed to survive. Thus 2 animals lived for 14 and even for 40 days and longer with 757 and 703 micrograms respectively; the first died of heat stroke and the other after the addition of stilbestrol (see below).

In groups II and III, with 852 to 1250 micrograms per day, only 2 out of 13 died before the 12th day and 1 on the 14th day. The others survived up to 162 days. As indicated in the notes for Table 2, the

TABLE 3. GUINEA PIGS TOTALLY AND PARTIALLY ADRENALECTOMIZED RECEIVING AOP AND STILBESTROL

Group	No. of animals sex	Loss of weight until death, gm.	AOP alone		AOP plus stilbestrol		Weight of adrenal fragment, mg.	Death
			Survival, days	Absorption per day microgm.	Survival, days	Absorption stilbestrol per day microgm.		
I	6* ♂	40–135	33	703–1250	2–14	20–53	0	Spontaneous
II	1* ♀	50	33	1000	9	4	0	Spontaneous
III	1 ♀	90	41	776	66	18	35	Spontaneous
IV	3 ♀	Gained weight	38	1105–1187	91	22–28	41–48	Sacrificed

* Included in Table 2.

survival of these animals was terminated for other experimental purposes. The animals which survived longest (153 and 162 days with 954 and 1168 micrograms of AOP per day) were killed with gas in order to determine whether the survival was due to glandular remains *These were not found.*

According to the results obtained, the survival of the adrenalectomized guinea pig can be obtained with 700 micrograms more or less of AOP per day. But this amount does not always assure the maintenance of all animals since even with 940 micrograms the death of one animal occurred within less than 7 days. The survival of all the animals can be obtained with 1000 micrograms per day. Thus of 12 animals (groups I and II) which received less than 1000 micrograms per day, 7 died within less than 7 days; on the other hand of 9 animals which absorbed more than 1000 micrograms (group III) none died within this period.

Acetoxypregnenolone and stilbestrol. In Table 3 are shown 11 animals which lived for more than 1 month with the implant of AOP and in which a pellet of 40% stilbestrol with cholesterol was implanted subsequently

The 6 males of group I and the female of group II all died unexpectedly not longer than 14 days after the implantation of the estrogen. The quantity of estrogen absorbed per day was less than the

amount frequently given to castrated guinea pigs for experimental purposes.

In 4 females included in groups III and IV of the table death did not occur as in the previous cases. For this reason we made a new implantation of stilbestrol. The animal of group III showed from that time a progressive fall in weight until its death 31 days after the second implantation of stilbestrol. At autopsy an adrenal fragment weighing 35 mg. was found. The 3 animals of group IV continued to live until 91 days after the first implantation of stilbestrol. They were sacrificed and in all of them adrenal fragments were found at autopsy. Since these 3 animals of group IV are the only ones which withstood the estrogen, we believe that the death of the animal of group III was due as much to the lower absorption of AOP as to the lower weight of the adrenal fragment.

DISCUSSION

The removal of the adrenals of the guinea pig is a laborious undertaking. But with sufficient mastery of the technique the gland can be completely extirpated, and at the same time one can look for the existence of accessory adrenal bodies, which are very seldom present. In those of our operated guinea pigs which survived without treatment, the adrenal fragment was seen immediately with the naked eye. We have seen glandular fragments with the naked eye even when they did not weigh more than 3 mg. In the rat, on the contrary, in order to make certain of the total extirpation of the adrenal it is necessary to study the region microscopically. For this reason the guinea pig has been an excellent test animal for our studies of the role of the adrenal cortex in the steroid equilibrium.

Clark (1941) kept the adrenalectomized guinea pig alive for 5 months with 0.5 to 1 mg. per day of DCA adding sodium chloride to the food. We have made the guinea pig survive for varying lengths of time with as little as 175 micrograms per day of DCA. It is true that this amount of DCA and even greater amounts do not necessarily assure the survival as is shown by the results in group II of Table 1. But it is evident that we obtained survival with *smaller* amounts of DCA than did Clark. This is probably due to the fact that we administered DCA by subcutaneous implantation of pellets and not by injection.

On the other hand it is possible that amounts of DCA greater than 250 micrograms were toxic to our adrenalectomized guinea pigs. It is worthy of note that *all* of the 6 animals of group II in Table 1 which died in the course of 4 to 11 days received relatively large amounts. It would be difficult to say whether our results are sufficient to show that amounts of DCA greater than 250 micrograms are toxic *when absorbed from a subcutaneous pellet*, as is suggested particularly by the diagram (fig. 1). On the other hand, it is clear from Tables 2

and 3 that this did not occur with AOP. The daily requirement of the adrenalectomized guinea pig is approximately 1 mg. of AOP. With a greater dose death never occurred before the 10th day, as it did in 5 animals receiving over 250 micrograms of DCA. Certain results from the recent literature are worth mentioning here. Thus Selye (1941) found that the administration of 35 mg. of DCA by subcutane-

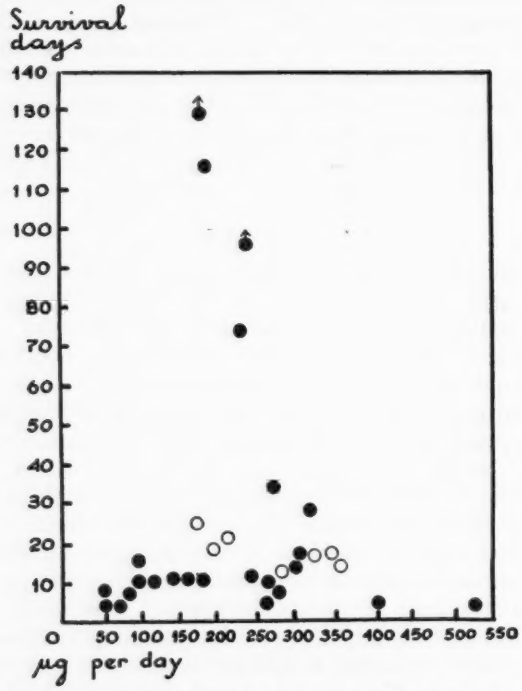

FIG. 1. 32 adrenalectomized guinea pigs treated with DCA given by subcutaneous implantation. Abscissa: quantity of steroid absorbed per day. Ordinals: days of survival. ● Animals dying with DCA present; ♠ animals dying after exhaustion of DCA; ○ animals dying after removal of DCA.

ous injection causes the death of the rat in 6 hours, while 50 mg. of pregnenolone administered under the same conditions produces no toxic effect. Despite the large variations in the length of survival which we observed in our animals, we have thought that something similar may have happened. Furthermore Swingle and Remington (1944) established the fact that the loss of salt and of water are not the only factors to which the death of adrenalectomized animals may be attributed; the dog can die with levels of blood electrolytes above normal. We have not studied the blood electrolytes in our animals; but one may ask whether the blood electrolytes behave in the same

way in adrenalectomized animals which survive with DCA and with AOP respectively.

Cleghorn (1943) demonstrated that in the dog the ratio of DCA to AOP required for survival is 1:6. Our findings in the guinea pig permit us to state this ratio as about the same, *i.e.*, 1:5.

From the point of view of the anti-estrogenic auto-defense exercised by the adrenal cortex, we believe that our results with the administration of stilbestrol to animals which had lived longer than 1 month with AOP are of great interest. As we have pointed out, all of these animals died 2 to 14 days after the action of the estrogen began, although the action of the AOP continued; the only exception was in those animals in which the adrenalectomy proved to be only partial. Our results agree with the fact that the survival of the adrenalectomized rat and mouse is shortened if natural or artificial estrogen is given after the ablation (Cavanaugh and Gaunt, 1937; Schachter et al., 1937; D'Amour and Funk, 1938; Gaunt et al., 1938; Selye and Masson, 1939; Pfeiffer and Hooker, 1940). But the toxic action of the estrogen does not occur if the animal is treated with estrogen before adrenalectomy (Cramer and Horning, 1939). Williams and collaborators (1945) observed that the estrogen (stilbestrol dipalmitate) caused marked weakness, anorexia and nausea in two addisonian patients. On the other hand Davis and Jones (1943) found that the estrogen prolongs the survival of adrenalectomized rats kept alive by DCA and exposed to low pressure.

The well-known fact that the adrenal gland increases in size when estrogen is injected is also worthy of mention (see the review of Tepperman, Engel and Long, 1943).

In our animals which were adrenalectomized and afterward treated with stilbestrol, the presence of a glandular fragment was sufficient to prevent the death of the animal. This protective function is related to the weight of the fragment. We have not studied the question whether larger amounts of AOP than we have used in our experiments would be able to prevent the toxic action of stilbestrol in the guinea pig, or the question of the behavior of DCA in this respect.

SUMMARY

Desoxycorticosterone acetate supplied to the adrenalectomized guinea pig by a pellet implanted in the subcutaneous tissue can keep the animal alive when it is absorbed in amounts of at least 175 micrograms per day.

This amount, however, and even larger amounts of DCA do not guarantee the survival of all animals.

The amount of DCA absorbed from a subcutaneous implant which permits the adrenalectomized guinea pig to survive is several times less than the amount needed when DCA is administered by injection.

With acetoxypregnenolone, the adrenalectomized guinea pig can

be maintained by approximately 1 mg. per day absorbed from a subcutaneous implant.

Both with DCA and with AOP we succeeded in maintaining the adrenalectomized guinea pigs for 4 to 5 months.

When stilbestrol in the form of a subcutaneous pellet was given to adrenalectomized animals which had been maintained with AOP for several weeks, they died in 2 to 14 days. Death did not occur in animals retaining an adrenal fragment which at autospy weighed approximately 40 mg., *i.e.*, probably less than 10 per cent of the normal weight of the adrenal.

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ADRENAL FUNCTION IN PREGNANCY[1]

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RELATIVELY LITTLE is known regarding the role of the adrenal in human pregnancy. Earlier investigators had noted, by direct observation that the adrenal gland undergoes definite hypertrophy during pregnancy, and more recently methods have been developed whereby adrenal activity can be investigated indirectly by the measurement of substances excreted in the urine which are derived from the metabolism of adrenal hormones. The adrenal gland has various functions, salt and water metabolism, carbohydrate and protein metabolism, and androgenic activity which may be associated with the different types of steroids which it elaborates. Many of the steroids isolated from urine have been considered as metabolites of these hormones. At present there are two groups of adrenal metabolites which are readily capable of assay by laboratory methods, the urinary 17-ketosteroids and the corticoids. While the 17-ketosteroids are considered to be associated with the androgenic activity of the adrenal, the urinary corticoids on the other hand possess the property of affecting carbohydrate metabolism. The excretion and relationship of these two types of adrenal metabolites have been reported in the normal individual by Venning and Kazmin 1946, and in pathological cases by Venning and Browne 1946.

With regard to the excretion of androgens and 17-ketosteroids in pregnancy, the fo lowing observations have been made. By biological assay Dingemanse, Borchardt and Laqueur (1937) found no increase in androgenic activity in the urine of pregnant women between the 6th and 8th month and Hain (1939) reported that pregnant women excreted less androgen than the non-pregnant. Pearlman and Pincus 1943, found that the 17-ketosteroids in human pregnancy did not differ significantly from those of non-pregnancy. In 1943, Samuels, Evans and McKilvery reported findings on the 17-ketosteroid excretion in a pregnant woman suffering from Addison's disease. They found a rise in the 6th month of pregnancy at that time at which the foetal adrenal begins to show some development and suggested that these ketosteroids were derived from the foetal adrenal. Following

Received for publication July 29, 1946.

[1] Supported in part by grants from the Associate Committee on Medical Research, National Research Council of Canada and the Hutchison Fund, Faculty of Medicine, McGill University, Montreal, Canada.

parturition the ketosteroid excretion dropped to its original low level. Burrows, MacLeod and Warren (1942) attempted to correlate the titer of excretion of ketosteroids in pregnancy with the sex of the foetus. They were unable to find a significant difference between the ketosteroid content of urines from pregnancies bearing a male foetus and those bearing a female foetus.

More recently Dobriner and coworkers (1943) in their analysis of pregnancy urine by means of chromatographic procedures found the androsterone decreased and some reduction in the etiocholanone fraction. The α ketonic fraction also contained relatively large amounts of pregnanolone and allo pregnanolone. Pregnanol-3(α)-one-20 had previously been isolated by Marker and Kamm (1937) from human pregnancy urine and allopregnanol-3(α)-one-20 by Marker *et al.* (1937) from the same source. Another stereoisomer allo-pregnanol-3(β)-one-20 was isolated by Pearlman *et al.* (1942) from this source.

A large number of investigations have been carried out on experimental animals with regard to the relationship of the adrenal to the gonads, both in the pregnant and non-pregnant state. Variations in adrenal size with the oestrus cycle have been observed in female rats and mice by several workers and striking increases in adrenal size in late pregnancy and especially in the puerperium may occur in rats, guinea pigs and rabbits. The administration of oestrogens has a markedly stimulating influence on pituitary and adrenal size. This subject has been extensively reviewed by Parkes (1945) and by Tepperman, Engel and Long (1943). In the present study an attempt has been made to evaluate adrenal function in human pregnancy by measuring the excretion of corticoids and ketosteroids throughout gestation. The excretion of pregnanediol, oestrogens and gonadotropins have also been followed in many of the cases. A preliminary report of these findings has been presented at the 10th Conference on Metabolic Aspects of Convalescence (1945) held under the auspices of the Josiah Macy Jr., Foundation.

EXPERIMENTAL METHODS

A 48 hour amount of urine was collected at varying intervals throughout pregnancy, usually every 3 to 4 weeks, and in most of the cases specimens were obtained following parturition. The urine was kept in a cool place during the collection. It was then divided into various aliquots for the following determinations.

Corticoids. The urinary corticoids were assayed by means of the method of Venning, Kazmin and Bell (1946) which is a bioassay especially devised for measuring small amounts of adreno-cortical substances. It is based upon the ability of these hormones to cause deposition of glycogen in the livers of adrenelectomized mize. The results are expressed in terms of glycogenic units, one unit being equivalent to the activity of one microgram of 17-hydroxy-11-dehydrocorticosterone.

Ketosteroids. The neutral urinary steroids obtained after acid hydrolysis of the urine were separated into ketonic and non-ketonic fractions by means of Girard's reagent T. The micromethod of Pincus and Pearlman (1941) was followed. The β-hydroxy fraction was separated by precipitation with digitonin. Color determinations were carried out on the total neutral ketonic fraction and after removal of the digitonin precipitable ketones. The amount of the latter fraction was obtained by difference. These two fractions were assayed by two different colorimetric methods, first by the method of Holtorff and Koch (1940) a modification of the Zimmermann reaction using dinitrobenzene as reagent for developing the color and then by the method of Pincus (1943) using antimony trichloride as reagent. The ketosteroids by these two methods will be referred to as ketosteroids (DNB) and keosteroids ($SbCl_3$). The Zimmermann reaction (DNB) measures not only 17-ketosteroids but also the steroids such as 3-ketosteroids and 20-ketosteroids. The latter have been shown to be present in pregnancy urine. The Pincus reaction ($SbCl_3$) does not include the 20-ketosteroids.

Pregnanediol. The Venning (1938) procedure for extraction of sodium pregnanediol glucuronidate from unhydrolysed urine was followed. Values are expressed as milligrams of pregnanediol excreted per 24 hours.

Oestrogens. The oestrogens were extracted with ether after hydrolysis with zinc and hydrochloric acid (Smith and Smith 1937) and assayed by the colorimetric method of Venning *et al.* (1937).

Gonadotropins. The gonadotropins were concentrated by the method of Katzman and Doisy (1934) and assayed on 21 day old hooded rats. The animals were injected subcutaneously twice daily for 3 days and then vaginal smears were taken once daily for the next 3 consecutive days. A full squamous response was considered as a positive result. The unit was defined as the smallest amount necessary to produce this result.

REPORT OF CASES

Nine cases of pregnancy were studied. The results of the different assays are charted in Figs. 1 to 8. In all cases the time of pregnancy is calculated from the first day of the last actually occurring menstrual period.

Case 1, S. aged 25 years, primipara—had a normal pregnancy and delivery. Studies were begun on the 110th day. The corticoid excretion remained within the normal range until the 200th day varying between 40 and 56 glycogenic units. After that time it rose reaching a peak of 160 units at the 260th day, then declined to 96 units on the 273rd day. A female child was delivered on the 296th day.

The ketosteroid (DNB) excretion which was within the normal range (9.5 to 13.2 mg.) until the 200th day increased gradually reaching a value of 20.3 mg. per 24 hours near term. The ketosteroid ($SbCl_3$) excretion on the other hand remained at a fairly constant level throughout pregnancy (8–12.5 mg. per 24 hours). The β ketonic fraction varied from 1.3 to 2.4 mg. per 24 hours. The excretion curves of pregnanediol and oestrogen were essentially normal.

Specimens were collected on the 3rd and 4th days post-partum.

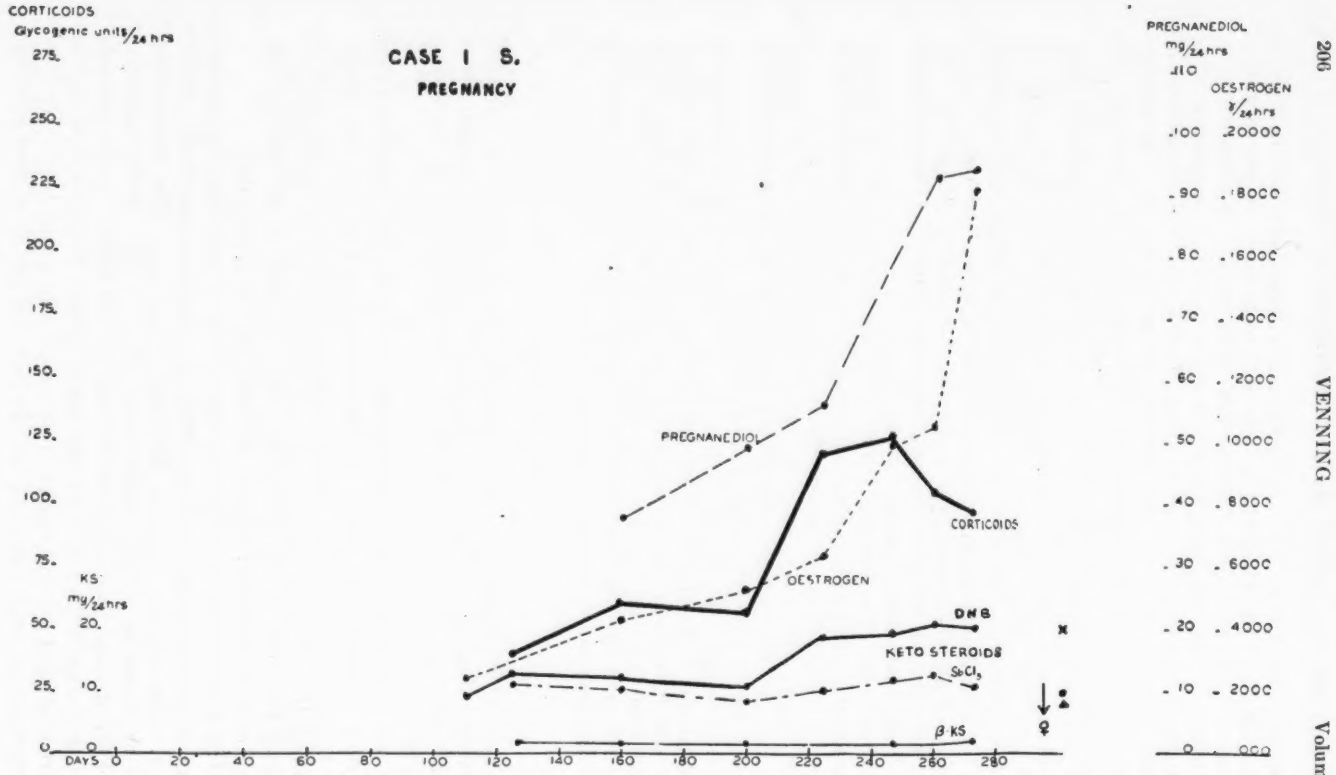

FIG. 1, CASE 1. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. The arrow denotes the time of delivery. X = corticoid excretion; ● = ketosteroids DNB and ▲ = ketosteroids ($SbCl_3$) after delivery.

The corticoid excretion had fallen to 50 glycogenic units and the ketosteroids were 9.4 mg. (DNB) and 7.5 mg. per 24 hours ($SbCl_3$) respectively.

Case 2, G., aged 22 years, primipara—normal pregnancy and delivery. Studies were begun on the 114th day. The corticoid excretion at this period was at a level of 228 units. It had decreased to 33 units by the 163rd day then rose again and remained at a high level until term. A female child was delivered on the 275th day. On the 3rd and 4th days post-partum the corticoid excretion had fallen to a normal level of 38 units. The ketosteroid ($SbCl_3$) excretion remained at approximately the same level throughout pregnancy, the ketosteroids (DNB) rose after the 193rd day reaching a value of 21.6 mg. per 24 hours on the 264th day. After delivery the values were 10.5 mg. (DNB) and 9.0 mg. per 24 hours ($SbCl_3$) respectively. No change in β ketosteroids were observed. Pregnanediol and oestrogen excretion were normal except for one low pregnanediol value on the 220th day.

Case 3, I., aged 24 years, primipara—normal pregnancy. On the 80th day of pregnancy the corticoid excretion was 90 units per day. It remained between 40 and 78 units until the 158th day then rose abruptly reaching a peak of 375 units on the 215th day and remained at a high level until term. A female child was delivered on the 278th day. On the sixth post-partum day the corticoid excretion was 62 units per day. The ketosteroid excretion curves were similar to those observed in the first two cases, the ketosteroids ($SbCl_3$) remaining at a fairly constant level while the ketosteroids (DNB) began to increase around the 186th day, finally reaching the level of 26 mg. per 24 hours. After delivery the values were 8.0 and 9.5 mg. per 24 hours respectively. The digitonin precipitable ketosteroid fraction varied from 1–2.0 mg. per 24 hours. The oestrogen excretion was within normal limits, but the pregnanediol values were on the low side in the latter part of pregnancy.

Case 4, H., aged 28 years, had had two pregnancies. The first pregnancy terminated at 7 months. In the second pregnancy, the membrane ruptured on the 120th day, and premature labour contractions occurred in the seventh month, between the 200th and 225th days. Also during the period between the 130th and 150th days the patient was in bed with a severe cold. Assays were begun on the 75th day. The corticoid excretion which was 150 units at that time fell to normal levels then after the 150th day increased rapidly. The curve showed 2 peaks at the 182th day and the 247th days when the corticoid excretion reached the high levels of 280 and 360 units respectively.

The ketosteroid excretion which was higher at the beginning of the study, 16.7 mg. (DNB) and 13.1 mg. ($SbCl_3$) dropped to a lower level between the 100th and the 167th day, then increased again up to the 206th day. At that time the ketosteroid ($SbCl_3$) curve flat-

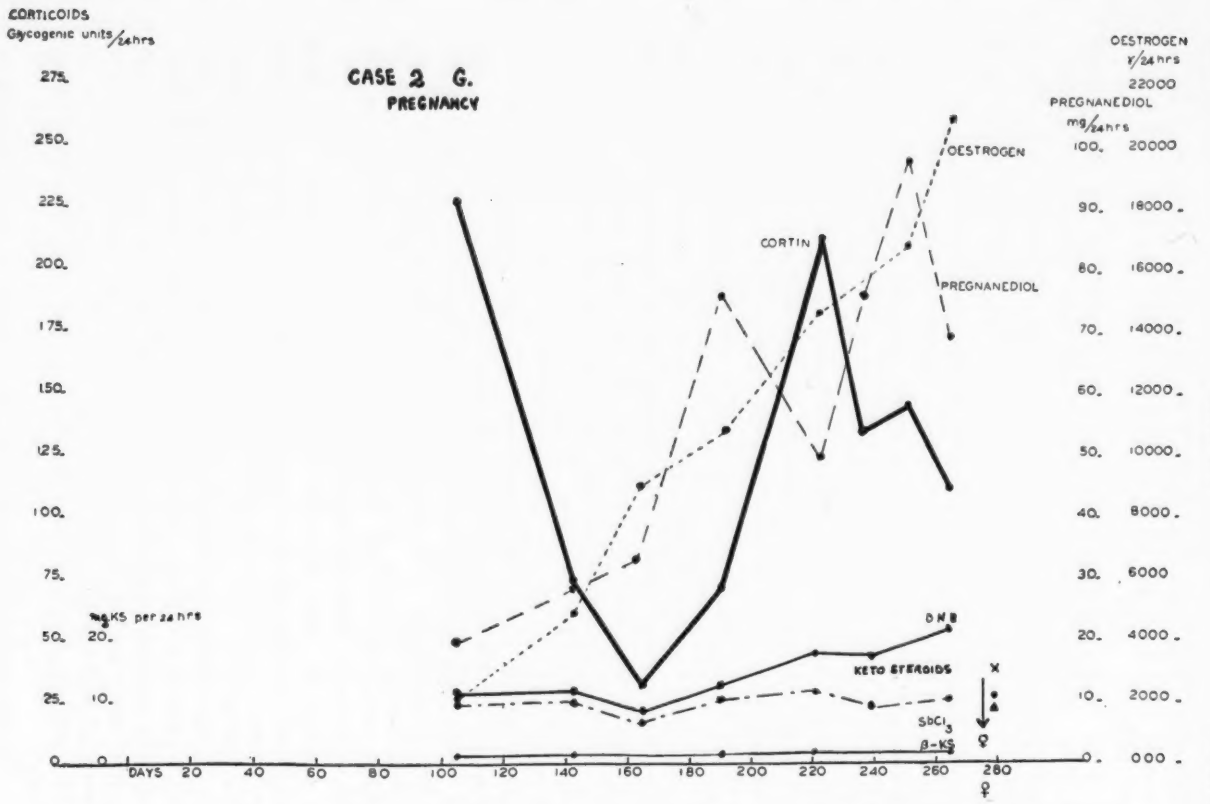

FIG. 2, CASE 2. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. The arrow denotes the time of delivery. X = corticoid excretion; ● = ketosteroids DNB and ▲ = ketosteroids ($SbCl_3$) after delivery.

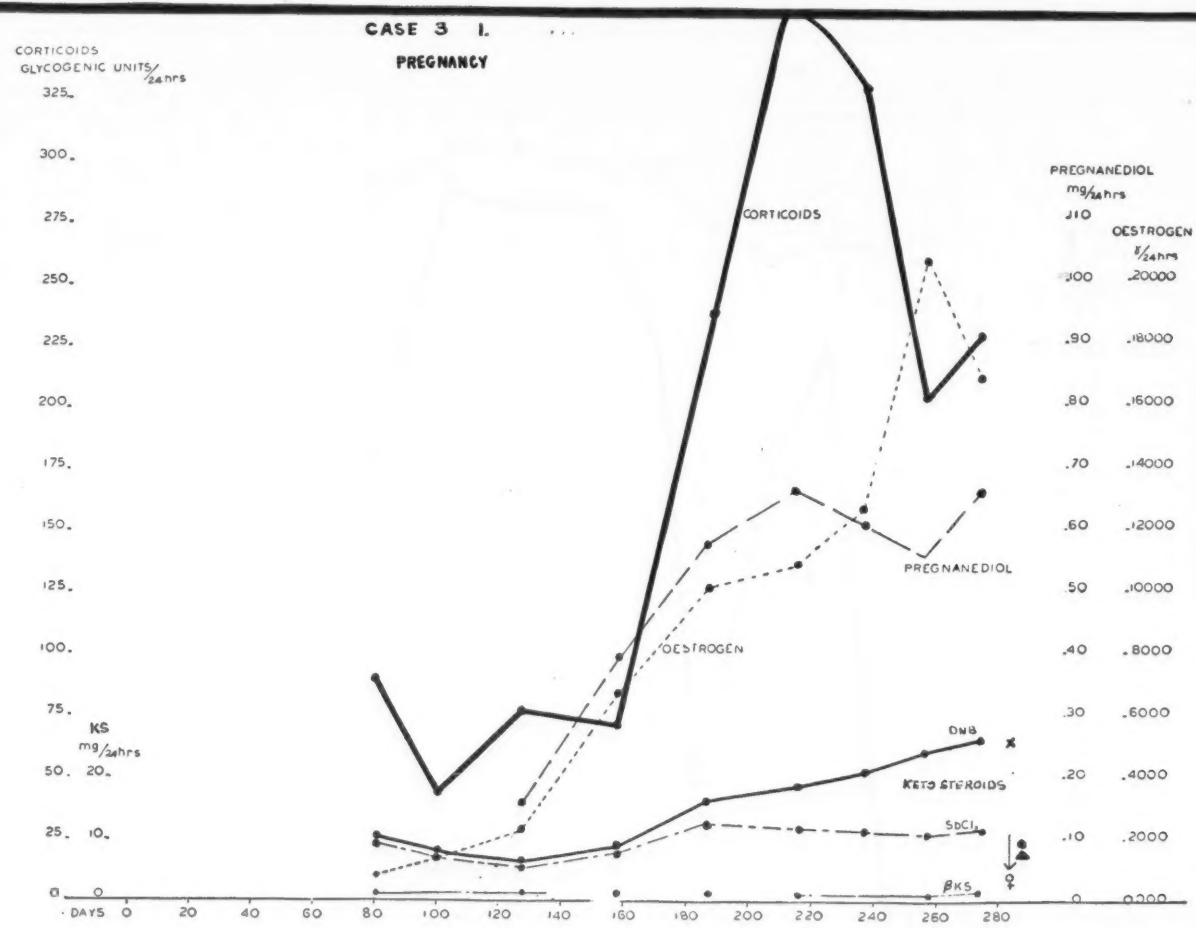

FIG. 3, CASE 3. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. The arrow denotes the time of delivery. X = corticoid excretion; ● = ketosteroids DNB and ▲ = ketosteroids ($SbCl_3$) after delivery.

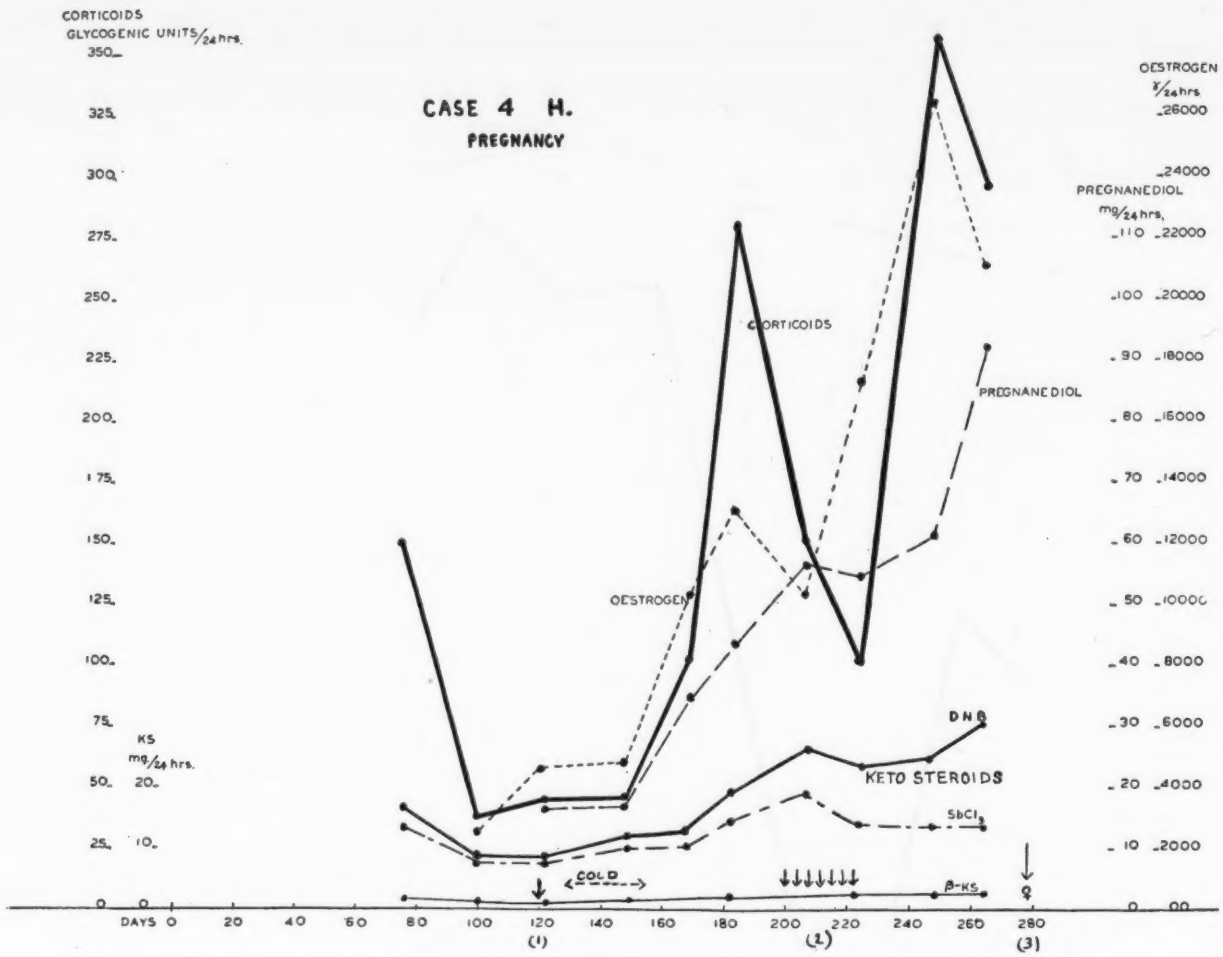

FIG. 4, CASE 4. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. The arrow denotes (1) rupture of membranes, (2) spontaneous contractions, (3) delivery.

tened out at its original level while the ketosteroids (DNB) rose again reaching the high values of 31 mg. per 24 hours at the 265th day.

Both the pregnanediol and oestrogen values which had remained stationary during the period of illness rose sharply afterwards but later showed a drop at the time of the premature contractions, however, they increased again in the 8th and 9th months. A female child was born on the 278th day. No assays were done post-partum.

Case 5, P., aged 35 years, had had 3 normal pregnancies. The last two pregnancies were followed. They are shown in Fig. 5, as (1) and (2). There was an interval of 2 months between them. *First pregnancy:* Only the corticoids and ketosteroids (DNB) were assayed in this pregnancy. On the 170th day the corticoids were 100 units, they rose rapidly reaching a value of 250 units on the 270th day. The ketosteroids increased from 11.5 mg. per 24 hours on the 170th day to 27 mg. per 24 hours on the 270th day. *Second pregnancy:* In this pregnancy studies were begun much earlier. On the 67th day, the corticoid excretion was 92 units. It then dropped to normal levels and increased again after the 170th day. The ketosteroid (DNB) curve was similar to the previous one, increasing in value towards the end of pregnancy, while the ketosteroid ($SbCl_3$) showed little change throughout. The β ketosteroids varied from 0.6 mg. to 2.0 mg. per 24 hours. The pregnanediol and oestrogen values were normal. A Caesarean section was performed on the 273rd day.

Case 6, C., aged 30 years, had a miscarriage 2 years previously at 3 months. The second pregnancy was normal. The patient has a history of irregular menses. Aschheim-Zondek tests done on the 36th and 41st days from the last menstrual period were negative. By the 60th day however, the gonadotropins assayed 3200 rat units per 24 hours, reaching a peak of 51,000 units on the 74th day. On the 117th day they assayed 40,000 units and had fallen to 10,500 units by the 136th day. Between the 41st and 100th days the corticoids excretion remained within the normal range. After that time it increased and on the 280th day the output had reached the level of 276 glycogenic units. Due to the delayed ovulation, delivery did not occur until the 302nd day. A male child was born. Three weeks later the urinary corticoids were 47 units per 24 hours. The ketosteroids ($SbCl_3$) remained at approximately the same level throughout pregnancy, there was however, an increase in the ketosteroids (DNB), after the 240th day. The β ketosteroids did not change. The pregnanediol and oestrogen values were normal.

Case 7, M., aged 32 years, gravida III, para 0. The patient had had 2 previous miscarriages over a period of 5 years. In the third pregnancy, bleeding occurred on the 64th day. 40 mg. progesterone was given orally from the beginning of pregnancy. The pregnanediol excretion was within normal limits from the 49th to the 62nd day. It declined to 9.6 mg. at the time of bleeding, but increased after that

FIG. 4, CASE 4. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. The arrow denotes

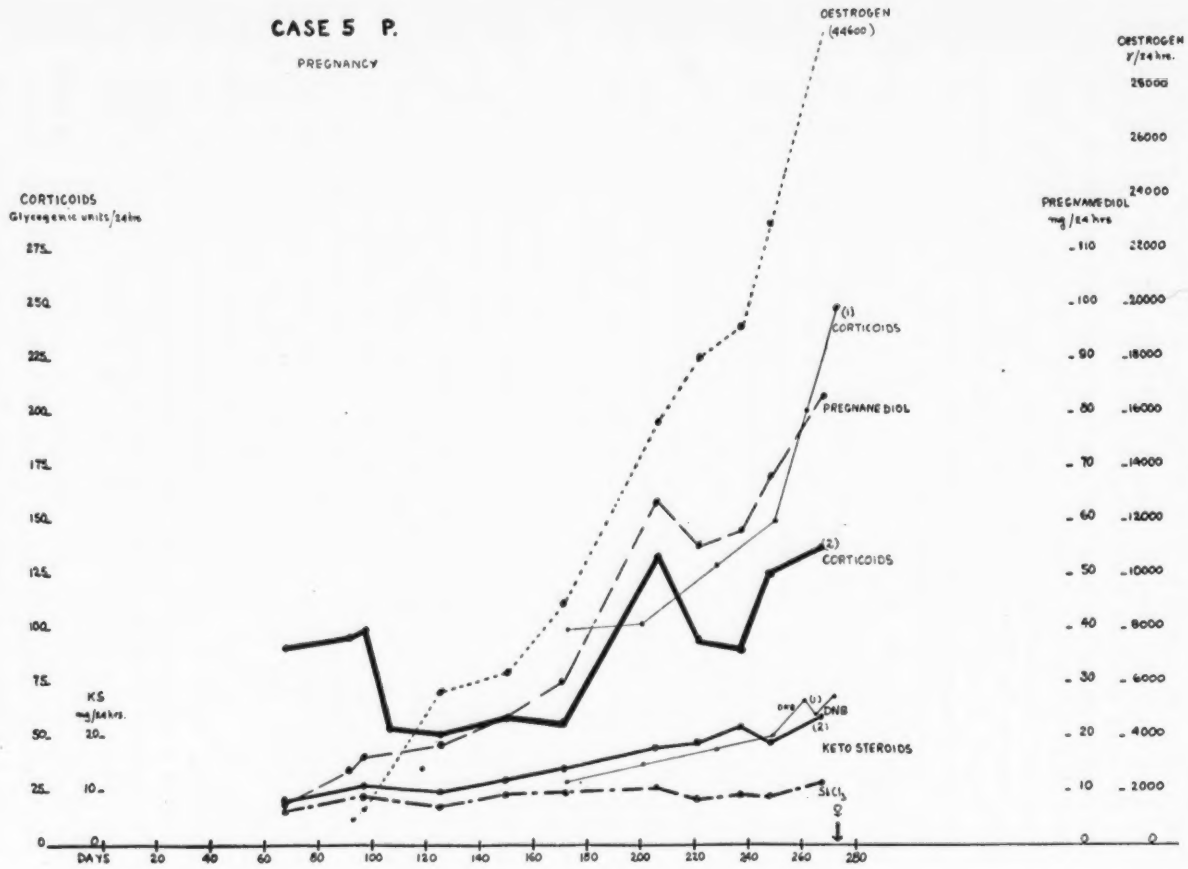

FIG. 5, CASE 5. Excretion of urinary corticoids, ketosteroids, pregnanediol and oestrogens in pregnancy. (1) first pregnancy. (2) second pregnancy. The interval between the pregnancies was 2 months. The arrow—Caesarean section in the second pregnancy.

(2) second pregnancy. The interval between the pregnancies was 2 months. The arrow—Caesarean section in the second pregnancy.

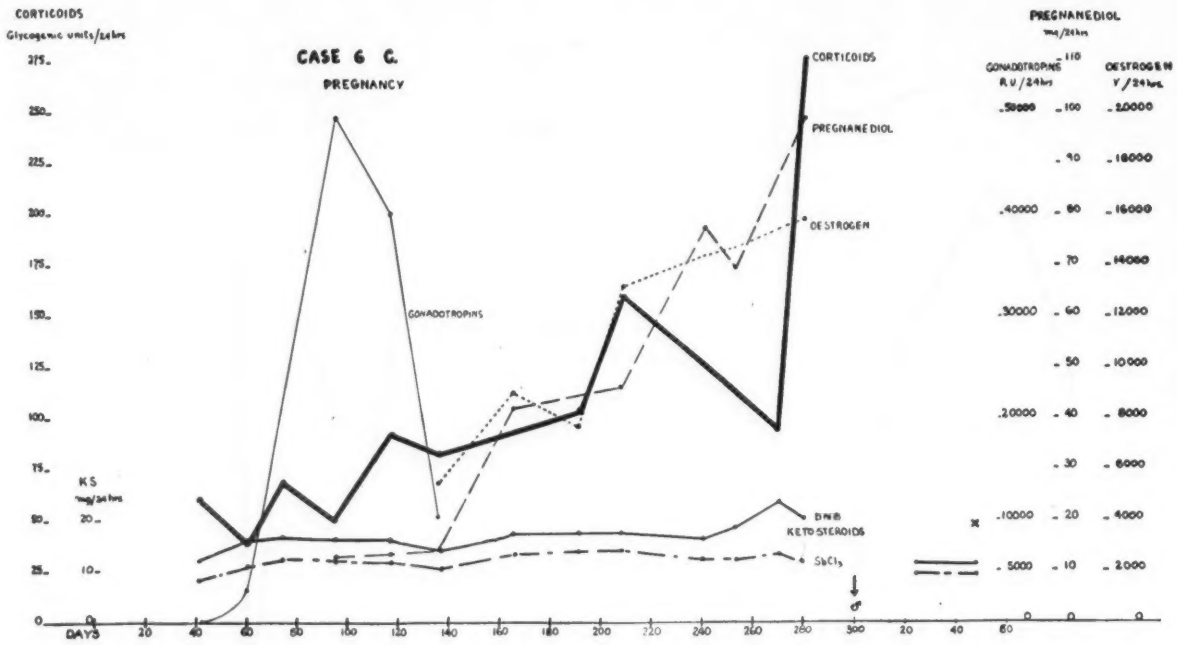

FIG. 6, CASE 6. Excretion of urinary corticoids, ketosteroids, pregnanediol, oestrogens and gonadotropins in pregnancy. The arrow denotes time of delivery. X = corticoids after delivery.

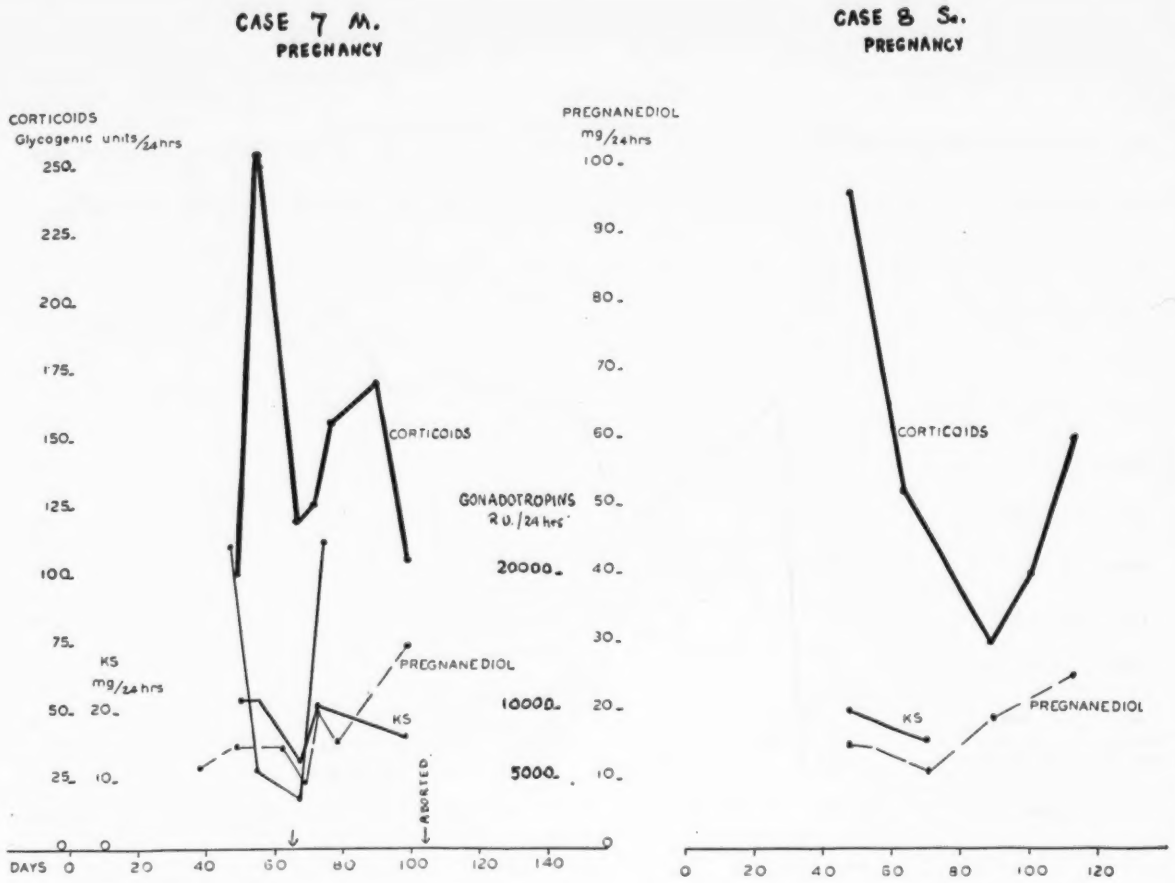

FIG. 7, CASE 7. Excretion of urinary corticoids, ketosteroids, pregnanediol and gonadotropins in an early pregnancy case, which aborted. CASE 8. Excretion of urinary corticoids, pregnanediol and ketosteroids in early pregnancy.

finally reaching a value of 30 mg. on the 97th day. The gonadotropins which assayed 22,000 rat units on the 49th day, fell abruptly before bleeding occurred and before the drop in pregnanediol excretion, reaching the low value of 4000 rat units on the 65th day. When assayed again on the 75th day they had risen to 22,500 rat units. Severe bleeding occurred again on the 102nd day and the patient aborted the following day. The corticoids were high throughout this early part of pregnancy, ranging from 105 to 244 glycogenic units and the ketosteroids (DNB) also increased.

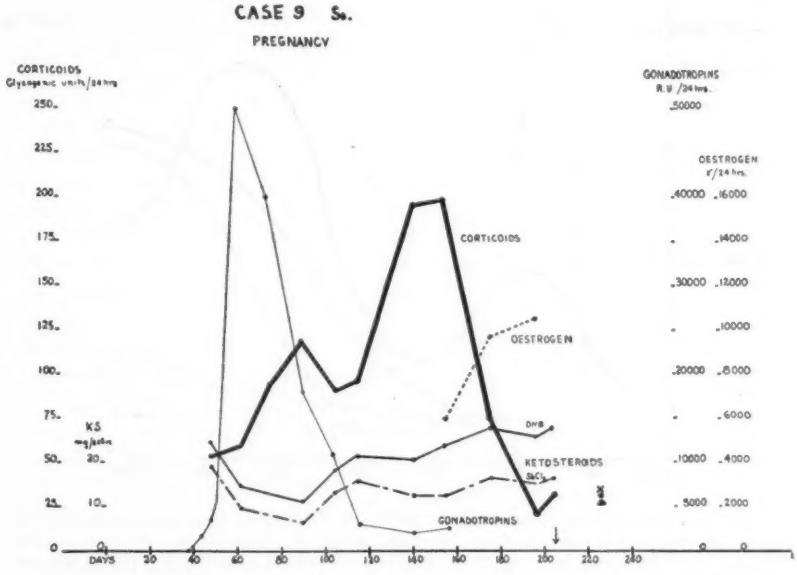

FIG. 8, CASE 9. Excretion of urinary corticoids, ketosteroids, oestrogens and gonadotropins in pregnancy. Arrow denotes time of bleeding and premature delivery due to a placenta praevia. X = corticoids; ● = ketosteroids (DNB) and ▲ = ketosteroids ($SbCl_3$) after delivery.

Case 8, Sch., aged 34 years, had had one normal pregnancy and one miscarriage. The third pregnancy progressed normally but the case was only studied in the first trimester. The pregnanediol excretion was normal, but the corticoids were increased during this period, ranging from 75 units to 238 units. The ketosteroids (DNB) assayed on the 47th and 64th days were 20 and 16 mg. per 24 hours respectively.

Case 9, Se., aged 27 years, had previously had one normal pregnancy. In the second pregnancy profuse hemorrhage occurred on the 202nd day caused by a placenta praevia which was followed by pre-

aborted. CASE 8. Excretion of urinary corticoids, pregnanediol and ketosteroids in early pregnancy.

mature delivery of a male child. The gonadotropins and oestrogens were within normal limits. The corticoid excretion was normal on the 48th and 62nd days, increased afterwards, reaching a value of 200 units on the 155th day. After this period it decreased and just before the bleeding occurred was at a normal level of 30 units. Twenty days after delivery it was still at this level.

The ketosteroids (DNB) were high in the beginning of pregnancy

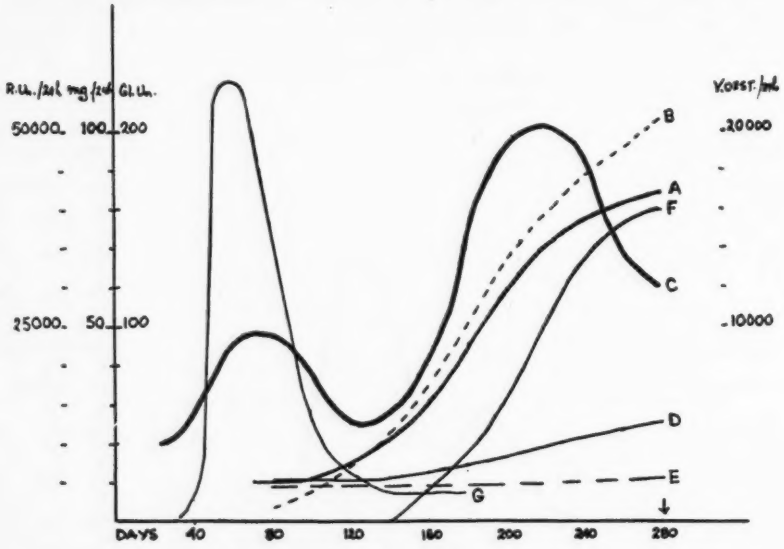

FIG. 9. Excretion rates of the various hormones in pregnancy (average values of the cases studied) A = pregnanediol; B = oestrogens; C = corticoids; D = ketosteroids (DNB); E = ketosteroids ($SbCl_3$); F = theoretical excretion curve for the pregnaneolones, assuming that the increase in difference between the two ketosteroid curves is due to these substances. G = Gonadotropins. Curve taken from a previous study on normal pregnancy cases.

fell from 24.6 to 11 mg. per 24 hours, then gradually increased to 27.5 mg. per 24 hours just before delivery. The ketosteroid ($SbCl_3$) curve followed the other curve at a lower level until the 115th day. After that time the values remained between 12 mg. and 16 mg. per 24 hours. Twenty days after delivery the values for ketosteroids (DNB) were 12.5 mg. and 11.0 mg. ($SbCl_3$) per 24 hours.

DISCUSSION

The important contributions made in recent years with regard to the metabolic changes produced by the adrenal cortical steroids show that the various functions of the adrenal, such as salt and water metabolism, carbohydrate, and protein metabolism, androgenic ac-

tivity, can be correlated with different groups of steroid hormones. Evidence is accumulating to indicate that these various functions are able to exert their influence independently of one another. The present research on the excretion of adrenal metabolites in pregnancy suggests that at least the corticoids, those compounds of adrenal origin,which have a specific effect on carbohydrate and protein metabolism, and the 17-ketosteroids can vary independently of one another. This is seen in Cushing's syndrome where the urinary corticoids are strikingly increased and the 17-ketosteroids are usually within the normal range. In cases of hirsutism the reverse is found, high 17-ketosteroids with normal corticoid excretion. In children the corticoid excretion has reached the adult level by the ages of 5–7 years, whereas the17-ketosteroids do not reach this level until a much later stage. In the present series of pregnancy cases a marked increase in excretion of corticoids was found with very little change in the 17-ketosteroids. The non-pregnant woman excretes from 25 to 65 glycogenic units of corticoids per day, the average being 39 units, while the average excretion of 17-ketosteroid is 11 mg. per 24 hours. It must be pointed out that the amounts of corticoids or active substances excreted in the urine are extremely small when compared to the 17-ketosteroids and probably only represent a fraction of the amount elaborated in the gland.

An analysis of the various excretion curves of corticoids in the different pregnancy cases revealed that they assumed a somewhat similar pattern. In Fig. 9 average excretion curves of the various hormones are presented for the 9 cases studied.

During the first trimester with the possible exception of Case 6, all showed an increased excretion of corticoids and in three of the cases, the excretion level was above 200 units. After this initial rise, the corticoid excretion returned to normal levels then between the 140th and 160th days began to rise again, reaching in some cases values over 300 units. Usually there was a falling off in output before parturition. These high excretions of corticoids observed in pregnancy are surprising as they are in the order of amounts found in the urine of individuals after severe damage or of patients suffering with Cushing's Syndrome and indicate that the activity of the adrenal must be greatly increased at this stage of pregnancy. The well known phenomenon of the rapid growth of the fetal adrenal cortex during the last trimester of prenatal life and its subsequent degeneration beginning at or just previous to birth, suggested the possibility that the fetal cortex might be contributing to the increased output of corticoids during the latter part of pregnancy. However, on examination of the urine obtained from infants in the first 4 days of life, little or no corticoid activity could be found. Within 2–4 days after delivery the excretion of corticoids in the mother was back to normal values.

Just what is the cause and function of this increase in adrenal

activity is not known. Investigations on experimental animals show that the administration of oestrogens have a markedly stimulating effect on the adrenals, also cyclic variation in adrenal size have been observed in many different species. In the human female, however, no cyclic variation in excretion of corticoids could be detected. While it is possible that the rise in excretion of oestrogens in late pregnancy may act as a damaging stimulus or toxic agent and thus cause adrenal hypertrophy, the earlier increase in corticoid excretion cannot be explained on these grounds. The first rise occurs at a time when there is a rapid increase in gonadotropins in the pregnant woman and may in some way be associated with this change. It appears to be more variable in the different cases than the subsequent rise in corticoids.

It has frequently been suggested that the adrenal might be one of the underlying factors in the production of late toxaemias of pregnancy. Parks in 1941 attempted unsuccessfully to correlate the incidence of toxaemia with the increase in weight of the fetal adrenals and in 1943 suggested that in the last trimester of pregnancy the extra cortical secretion from the fetal adrenal glands might cause a disturbance in electrolyte distribution and thus contribute to the production of oedema in the mother. While there is not at the present time any suitable method of measuring the excretion of metabolites derived from those adrenal substances having a specific effect on water and electrolyte metabolism, in the light of our present findings, the maternal adrenal rather than the fetal adrenal should be considered as the possible offender.

The earlier investigations using growth of the capon's comb as criterion of androgenic activity indicate that there is little change in the excretion of androgens throughout pregnancy, however, the more recent work of Dobriner and co-workers, using chromatographic methods of fractionation show that there is actually a marked decrease in the androsterone fraction, the compound thought to be mainly responsible for the androgenic effect in normal urine. When the Pincus color reaction is used for measuring 17-ketosteroids (this method includes all of the usually occurring urinary 17-ketosteroids with the exception of dehydroisoandrosterone) very little change in the excretion of 17-ketosteroids in pregnancy is noted, however, if the Zimmermann reaction is used a definite increase in output of ketosteroids in the latter part of pregnancy is observed in all the cases. This is due to the fact that other ketonic substances beside the 17-ketosteroids give a color with the Zimmermann reaction. It has been shown repeatedly that 20-ketosteroids and to a much lesser extent 3 ketosteroids also give a color in this reaction. Three 20-ketosteroids, stereoisomers of pregnanolone have been found to be present in pregnancy urine. They are pregnanol 3α one 20, allo pregnanol 3(β) one 20. These 20-ketosteroids give only approximately

$\frac{1}{5}$ of the colour of the 17-ketosteroids, in the Zimmermann reaction. If the difference in color index of the two methods may be attributed entirely to the increased excretion of pregnanolones in pregnancy then the amounts excreted are indeed surprisingly large, in late pregnancy this would amount to approximately 80 mg. per day of ketonic substances other than 17-ketosteroids (Fig. 9). This is almost equal to the average excretion of pregnanediol in the latter stage of pregnancy. Earlier observers have reported finding only small amounts of the pregnanolones in pregnancy, however, the recent work of Dobriner indicates that they are present in relatively large amounts, but no figures are given. The increase in ketonic substances appears to be almost entirely in the digitonin non-precipitable fraction which contains the α ketosteroids and non-alcoholic ketosteroids. Only very slight increases are noted in the digitonin precipitable or β-OH ketonic fraction in these studies. This is surprising because increased adrenal activity has usually been associated with an increase in the excretion of β-OH ketosteroids such as dehydroisoandrosterone or isoandrosterone. The increase in the excretion of ketonic substances which is probably due to the excretion of pregnanolones begins between the 140th and 160th days of pregnancy. This is the time at which the corticoids begin to increase again. The rise in pregnanediol excretion usually occurs at an earlier stage, between the 100th and 120th day.

The pregnanolones have generally been considered as being derived from the metabolism of progesterone, however, in the various metabolic studies reported only pregnanediol has been recovered in the urine following administration of progesterone. We have repeated these investigations giving large amounts of progesterone (5 gms.) orally to 2 women and have failed to find significant amounts of other metabolites besides pregnanediol. Whether progesterone is metabolized differently by the pregnant woman, giving rise to both pregnanediol and pregnanolone or whether the pregnanolones may be derived from the metabolism of adrenal substances is an interesting conjecture. The fact that the ketosteroids (DNB) continue to rise in late pregnancy at a time when there is frequently a falling off in corticoid excretion (Cases 2, 3, 9) might be considered evidence against the latter theory.

SUMMARY

The urinary excretion of glycogenic corticoids, ketosteroids, pregnanediol, oestrogens and gonadotropins has been followed in nine pregnancy cases.

The corticoids were assayed by the bioassay method of Venning, Kazmin and Bell and include those adrenal metabolites which have a specific effect on carbohydrate metabolism.

There is an initial rise in excretion of corticoids in the first tri-

mester of pregnancy which usually returns to normal levels by the 100–120th day. Between the 140th and 160th days the urinary corticoids increase again reaching relatively high values.

A falling off in excretion usually occurs in the last month and shortly after parturition the values are back to normal again.

The ketosteroids when measured by the Pincus reaction remain at the same level throughout pregnancy. When the same fraction is assayed by the Zimmermann reaction an increase in ketosteroid excretion is seen beginning between the 140th to 160th day. This increase in ketosteroids is attributed to the increased excretion of 20-ketosteroids. Only a slight increase is observed in the β-OH ketonic fraction.

The relationship of the excretion of adrenal metabolites to the excretion of oestrogens, pregnanediol and the gonadotropins is discussed.

ACKNOWLEDGMENTS

We wish to acknowledge with gratitude the kindness of Dr. M. M. Braunstein in permitting these studies to be carried out on some of his cases and also the valuable technical assistance of Mr. V. Kazmin, Miss M. P. Ripstein and Miss Y. Bedwell in carrying out the numerous assays.

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NOTES AND COMMENTS

STIMULATING THE SEX DRIVE OF BOVINE MALES IN ARTIFICIAL INSEMINATION

METHODS obliged to be used in artificial insemination do not in all cases develop erotization of the nervous system by internal secretions as hypothesized by Steinach (1910) to be necessary for courtship and copulatory behavior.

In the first year of this work at the University Farm, 14 bulls were admitted to the breeding ring. One of these animals was by far the most satisfactory breeding animal and 65 collections of semen were obtained from April 1, 1945 to May 24, 1946. Three bulls were necessarily removed for lack of sex drive and three others were irregular breeders. The animals were always given access to a sterile female in a breeding chute. She stood readily for the procedure but rarely showed any estrous manifestations.

The irregular bulls Nos. 101, 103 and 205 at first worked satisfactorily. Reduced sex drive and quality of semen was noticeable in a few months. Semen collections in some cases were almost water clear consisting only of accessory gland secretions. For periods of time the irregular bulls completely lost sexual libido and would pay no attention to the female. Hormone therapy by subcutaneous injections of equine gonadotropin and testosterone propionate were tried with unsatisfactory to negative results. No. 101 was returned to natural service and immediately became an active breeder.

The following experiments were then staged to test the psychical influence of a cow in estrus on the sex drive of Nos. 103 and 205.

On April 11, with a cow in estrus available at a distance No. 103 was brought out to the sterile female, showed the usual lack of interest and voluntarily returned to his pen. The genitals of this cow were then smeared with mucus from the vagina of the cow in estrus and the bull again admitted. More interest was now shown but no attempt to mount was exhibited. This sterile cow was then removed and the cow in estrus led alongside the pen containing No. 103, and stanchioned in the chute. Interest was at once exhibited as the cow passed the bull pen. On letting him into the breeding chute he mounted at once and serviced the artificial vagina actively. He dismounted but almost immediately (one minute) again mounted the animal and serviced the artificial vagina a second time. The procedure was repeated a third time in three minutes and this bull was then driven back into his pen with considerable difficulty.

No. 205 was then let out, immediately mounted the estrous cow, serviced the artificial vagina and repeated in three minutes. Both of these animals continued to manifest great interest in the estrous cow as she was removed from the breeding chute. In addition to sex drive increased quantities of

Received for publication July 8, 1946.

opaque, dense semen were ejaculated with a high concentration of very active sperm. The later despite efforts to collect from No. 103 had been previously unsuccessful since March 15, a period of 27 days.

On April 26, No. 103 was brought out to the regular non estrous cow and showed no interest. On May 3, with another estrous cow he serviced the artificial vagina three times in rapid succession and 8 mils. of high quality semen were collected.

On May 10, the genitals of the non estrous cow were smeared with a mixture of vaginal mucus and urine from an estrous cow. No. 103 was then let into the breeding chute, mounted the animal and serviced the artificial vagina twice in five minutes from which 8 mils. of semen were collected. This animal was succeeded by No. 205, which followed the same procedure and 5.5 mils. of semen were obtained.

Both of these animals on this date showed interest when the non estrous cow passed their pens. This may have resulted from the psychical effect of the presence of the cow in estrus on May 3. At this time neither bull was as active as on May 3 with the estrous cow.

On June 7, two other bulls were used on a cow in heat. No. 103, penned close by, manifested great interest. The latter animal was then let out to the non estrous cow, mounted vigorously, and serviced the artificial vagina twice.

No. 101 was returned to the breeding ring on May 18. He was successfully used on an estrous cow May 24, producing 8 mils. of good quality semen from one ejaculate. The following week, May 31, he failed to mount the regular non estrous cow.

SUMMARY

The small amount of data presented opens some interesting questions regarding sexual behavior in the male bovine of great importance in artificial insemination programs.

In this procedure the psychical effect on bulls of non estrous versus estrous cows is very great and may affect the efficiency of the ring and number of bulls required. In different species female manifestations of estrus vary, Stone (1939). In the bovine evidence presented would suggest that there is an odor from the female rather than her behavior that attracts and psychically stimulates the male under the conditions obtaining in an artificial insemination breeding chute. The effect of this is so marked that it immediately stimulates bulls to breed that otherwise appear to be impotent. The breeding act is vehement with copious ejaculation of semen of high quality. The assumption is justified that sperm are included from deep in the epididymus as compared to older cells which had accumulated in the ampulla. If an odor is responsible its origin has not been demonstrated. Body fluids or tissues containing it might be preserved. If such a product could be placed on a dummy or the genitals of a non estrous cow in a breeding chute and enhance sexual drive of males, efficiency of artificial insemination would be greatly benefited. The same result might be accomplished by the use of estrogens to have the regularly used cow in estrus on collection days. Cows naturally in estrus are frequently not available when semen collections have to be made. The experiments with these bulls indicate that the presence of

sufficient androgens did not necessarily produce sex drive and normal copulatory behavior.

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ASSOCIATION NOTICE

The 29th annual meeting of the Association for the Study of Internal Secretions will be held Friday and Saturday, June 6 and 7, 1947, in the Viking Room of Haddon Hall Hotel, Atlantic City, New Jersey, preceding the Centennial meeting of the American Medical Association.

Members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make your reservations directly with Chalfonte-Haddon Hall advising them of the accommodations you wish. Rates are as follows:

	Chalfonte	*Haddon Hall*
Single room with bath:	$ 6. 7. 9.	$ 7. 8. 10.
Double room with bath (without ocean view):	8. and 10.	10. and 12.
Double room with bath (side ocean view):	12.	14.
Double room with bath (ocean front):	14. and 16.	16. and 18.

Make your reservations now and avoid disappointment— emember, you can always cancel them at a later date.

Those wishing to present papers should send the title of the paper and four copies of a comprehensive abstract to the president, Dr. Fuller Albright, Massachusetts General Hospital, Boston, Massachusetts, at their earliest convenience. Abstracts submitted should be in proper form for printing in the program. Not more than the first two hundred words can be included in the printed abstract.

Further information regarding the meeting will be forthcoming at an early date.

NEW BOOK

ENCYCLOPEDIA OF ENDOCRINOLOGY, Section IV, The Ovary, Vol. VII, and Vol. VII (Ref.). By Hans Selye, M.D., Ph.D., Richardson, Bond and Wright, Montreal, 1946.

These two volumes, one of text with an extensive index, the other of references, are the second section of a great, living Encyclopedia of Endocrinology which is being compiled by Dr. Selye. He has long advocated that medical science would benefit from systematic reviews of various phases of various special subjects, and has undertaken this work to illustrate his idea, as an experiment in one field, Endocrinology. The success of this work's first section, the "Classified index of the steroid hormones and related compounds," led him to continue with the present section.

Selye has written a highly interesting *Foreword* in which he effectively portrays his vision of the great Encyclopedias he projects for each field of medicine. Unlike many other dreamers, he has offered a tremendous work as an example. The *Foreword* reveals some of the interesting developments along these lines that we may anticipate when the world's social organization is further perfected to support scientific effort.

The first of the volumes, containing the text, is printed in a loose-leaf system in order to minimize the expense of keeping the material up to date with supplements. It is stated that the new material will be issued in such a way that sections with new pages can be slipped into the place of the old. An elaborate system for numbering of pages is invented for this purpose. The illustrations are beautifully clear and are very numerous. The numerous references in this volume of text are cited in a system aimed to make the reading easy, yet to have the references easily sought.

The reference volume is not loose-leaf and is reproduced by a photographic ("photorepro") process directly from the manuscript typed by Electromatic typewriters. The author states that this was done in order to reduce the possibility of errors in transcribing. The result is a very legible and undoubtedly highly accurate product.

The references are listed in alphabetic order, and each has an identification number. The only sacrifice detected by the reviewer, is the omission of the final page of each reference. No doubt this would have been too much to expect in such a work, but, nevertheless would have added a certain benefit.

This Encyclopedia deserves to be included in every major reference library and in every laboratory of Endocrinology.

KENNETH WADE THOMPSON, M.D.